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(71) Applicant: **Biostest AG**  
**63303 Dreieich (DE)**

(72) Inventors:  
 • **Kistner, Steffen**  
**60599 Frankfurt/Main (DE)**

- **Schüttrumpf, Jörg**  
**63303 Dreieich (DE)**
- **Daufenbach, Jens**  
**55120, Mainz (DE)**
- **Herbener, Peter**  
**35274 Kirchhain (DE)**
- **Ungerer, Christopher**  
**63225 Langen (DE)**

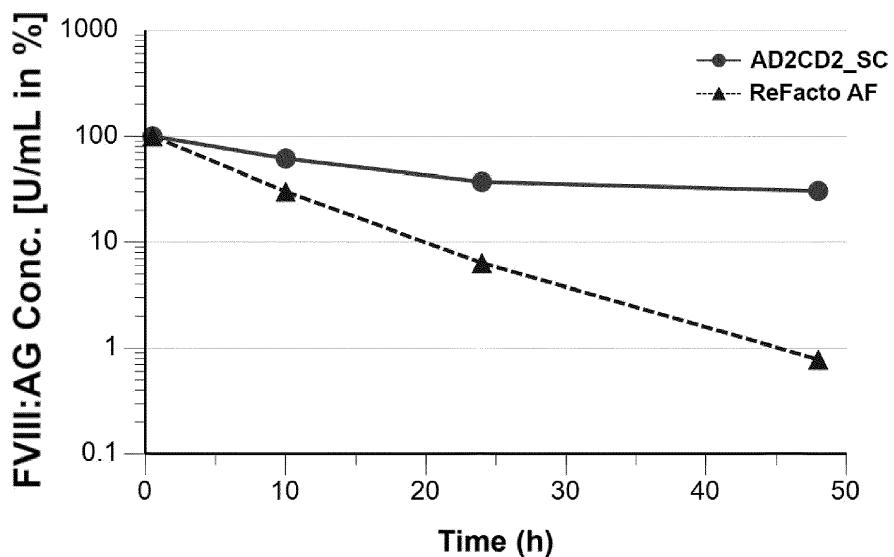
(74) Representative: **Moré, Solveig Helga et al**  
**Kroher Strobel**  
**Rechts- und Patentanwälte PartmbB**  
**Bavariaring 20**  
**80336 München (DE)**

## (54) FACTOR VIII PROTEIN WITH INCREASED HALF-LIFE

(57) The present invention relates to recombinant coagulation factors, in particular, recombinant Factor VIII (FVIII) proteins having an increased half-life. They comprise a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion. If

the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion. The invention also relates to nucleic acids encoding the proteins of the invention, methods of producing them and pharmaceutical compositions comprising any of these, wherein the pharmaceutical composition preferably is for use in treatment of hemophilia A.

Fig. 5



**Description**

**[0001]** The present invention relates to recombinant coagulation factors, in particular, recombinant Factor VIII (FVIII) proteins having an increased half-life. They comprise a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion. If the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion. The invention also relates to nucleic acids encoding the proteins of the invention, methods of producing them and pharmaceutical compositions comprising any of these, wherein the pharmaceutical composition preferably is for use in treatment of hemophilia A.

**[0002]** FVIII is an important co-factor in the coagulation cascade. Wildtype human FVIII is synthesized as a single chain consisting of 2351 amino acids and comprises three A domains (A1-A3), one B domain and two C domains (C1 and C2), interrupted by short acidic sequences (a1-a3). The first 19 amino acids are the signal sequence, which is cleaved by intracellular proteases, leading to a FVIII molecule of 2332 amino acids. The resulting domain structure is A1-a1-A2-a2-B-a3-A3-C1-C2. During post-translational modification, FVIII becomes glycosylated, sulfated and proteolytically processed. Sulfation is important for the extracellular interaction with different proteins, especially Thrombin and von Willebrand factor (vWF). It takes place on six tyrosines in the acidic regions a1, a2 and a3. Intracellular cleavage, by the serine protease furin, divides FVIII into a heavy chain (A1-a1-A2-a2-B) and a light chain (a3-A3-C1-C2). During this cleavage, parts of the B domain can be lost. Therefore, the light chain has a molecular weight of 80 kDa, whereas the heavy chain can be slightly heterogeneous, with a molecular weight around 210 kDa. The binding between heavy and light chain is not covalent, but mediated by the divalent metal ion Cu<sup>2+</sup> between the A1 and A3 domain.

**[0003]** In the circulation, FVIII is bound to vWF via the a3, C1 and C2 domain, which protects FVIII from early activation as well as degradation.

**[0004]** Upon activation, FVIII is cleaved by Thrombin at three positions, leading to a heterotrimer and loss of the B domain (heterotrimeric FVIIIa). The heterotrimer forms a complex with the activated coagulation Factor IXa and coagulation Factor X, and the light chain binds to a phospholipid bilayer, e.g., the cell membrane of (activated) platelets.

**[0005]** Hemophilia A mainly is a genetic bleeding disorder linked to the X-chromosome, occurring in 1 of 5000 newborn males. However, Hemophilia A can also occur spontaneously due to an auto-immune response against FVIII. Patients with Hemophilia A suffer from longer bleeding durations, spontaneous and internal bleedings, affecting their everyday life.

**[0006]** Hemophilia A patients are generally treated by administration of FVIII. Depending on the severity of the disease (mild, moderate or severe), treatment is on demand or prophylactic. Therapeutic FVIII products are either purified from human plasma (pFVIII) or the products are produced recombinantly in cell culture (rFVIII).

**[0007]** During the development of recombinant FVIII molecules for therapy, B-domain deleted FVIII molecules have been designed, because the B-domain is not important for the functionality of FVIII in clotting. This predominantly leads to a reduction in size. One of the most common B-domain deleted FVIII product is ReFacto® or ReFacto AF® produced by Pfizer. This FVIII variant lacks 894 amino acids of the B domain.

**[0008]** One issue with regard to FVIII substitution therapies is the relatively low in vivo half-life of the plasma-derived or recombinant Factor VIII proteins. There have been different approaches for enhancing FVIII half-life reaching from PEGylation to albumin incorporation, single chain molecules and Fc fusion.

**[0009]** WO 2014/070953 A1 relates to a method of reducing or decreasing the bleeding rate of a hemophilia patient by administering a long acting Factor VIII polypeptide, wherein the long acting Factor VIII polypeptide may be a fusion of the Factor VIII polypeptide and a heterologous moiety, which is an FcRn binding partner and may comprise an Fc region.

**[0010]** WO 91/01743 A1 describes a process for extending the half-life of a biologically active protein or peptide in vivo by covalently coupling said protein or peptide to a polypeptide fragment capable of binding to a serum protein, especially to serum albumin. Preferably, the albumin-binding fragment derives from streptococcal protein G or staphylococcal protein A. The generated fusion protein binds to serum albumin in vivo, and benefits from its longer half-life, thereby increasing the net half-life of the fused therapeutically interesting protein or peptide. WO 2005/097202 A2 generally mentions fusion proteins combining a therapeutic protein and a plasma protein in a single polypeptide chain, wherein such fusion proteins may provide clinical benefits in requiring less frequent injection and higher levels of therapeutic protein in vivo.

**[0011]** WO 2009/016043 A2 discloses an albumin binding polypeptide. WO 2010/054699 A1 discloses a capture molecule for modulation of pharmacokinetics and/or pharmacodynamics of a target having a biological function in a mammal, wherein said molecule comprises at least one albumin binding moiety.

**[0012]** WO 2011/101284 A1 describes Factor VIII fusion proteins. Reference to albumin binding moieties is made. Fusion partners are presumed to delay in vivo clearance of FVIII by interaction with serum albumin. Albumin binding polypeptides, such as the ABD1 polypeptide, are mentioned as examples for fusion partners. Suggested fusion proteins comprise four albumin-binding moieties (albumin binding domain - ABD) comprising ABD1, e.g. 4 x ABD1 in the B domain or 4 x ABD1 at the C-terminus of the FVIII light chain.

[0013] WO 2012/004384 A2 discloses an albumin binding sequence. It describes a fusion protein or conjugate with the albumin binding polypeptide, wherein the second moiety may be Factor VIII.

[0014] WO 2013/143890 A1 discloses a compound for oral administration comprising a moiety with desired therapeutic activity and a moiety that binds to albumin.

5 [0015] WO 2014/048977 A1 discloses a class of engineered polypeptides having a binding affinity for albumin. WO 2014/064237 A1 provides albumin binding domain binding polypeptides comprising an ABD binding motif. WO 2015/091957 A1 relates to a class of engineered polypeptides having a binding affinity for albumin, wherein the polypeptides have a high resistance to proteolytic cleavage.

10 [0016] In light of the state of the art, the inventors of the present invention addressed the problem of providing recombinant FVIII proteins with increased half-life, which preferably allow a more convenient substitution therapy for the patient, allowing administration intervals of more than three days or even more than one week or more.

[0017] This problem is solved by the present invention, e.g., by the claimed subject-matter.

15 Description of the invention

[0018] The inventors have constructed new FVIII variants having a longer in vivo half-life and excellent specific activity as evidenced by different biological activity assays. These proteins further have a high level of expression and a low profile of fragments and side products. Further advantages and preferred embodiments are explained elsewhere in this description. The inventors have found that a particular arrangement of albumin binding domains contributes to an increase in in vivo half-life.

[0019] The invention thus provides a recombinant Factor VIII protein comprising a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion. If the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion.

[0020] Factor VIII in complex with von Willebrand factor (vWF) has an in vivo half-life of about 12 hours. FVIII not associated with vWF is typically degraded much more rapidly.

[0021] Albumin has an in vivo half-life of about 19 days. By introducing at least two albumin binding domains in the FVIII sequence, it was possible to obtain a significant half-life prolongation. Different positions and different numbers of the albumin binding moiety have been tested in order to identify the optimal positions and numbers of integrated albumin binding moieties.

[0022] Without intending to be bound by the theory, it is believed that albumin binding to the FVIII protein of the invention through the albumin binding domains in the specific positions described herein is particularly effective in inhibiting breakdown of the FVIII protein of the invention. This appears to increase in vivo half-life more than association with vWF associated with native FVIII in blood.

35 FVIII

[0023] The skilled person understands the term FVIII (or Factor VIII) and is aware of the structure and biological 40 functions of wild type FVIII and typical variants thereof. Apart from the features specified herein, the FVIII protein of the invention may be designed as deemed appropriate and advantageous by the skilled person.

[0024] In particular, the Factor VIII protein of the invention should typically comprise all necessary portions and domains 45 known to be important for biological function. For example, preferably, the FVIII protein further comprises domains corresponding to, substantially corresponding to, and/or functionally corresponding to the A and C domains of wild type FVIII, especially to A1, A2, A3, C1 and C2 domains. It may further comprise additional portions and domains. For example, preferably, the FVIII protein further comprises an a1 domain between the A1 and the A2 domains and an a2 domain C-terminal to the A2 domain. For a double chain protein, on a separate chain, or for a single chain protein, C-terminal to said domain, and, optionally, C-terminal to the B-domain or a truncated B-domain and to at least one albumin binding domain, the FVIII protein comprises at least a truncated a3 domain. Before processing, the Factor VIII protein of the invention may also comprise a signal sequence. Thus, the heavy chain portion preferably comprises the domains A1 and A2, and typically comprises the domains A1-a1-A2-a2 or A1-a1-A2-a2-B. Preferably, the B-domain of the Factor VIII protein is at least partly deleted. The light chain portion preferably comprises the domains A3 and C1 and C2, and typically comprises the domains a3-A3-C1-C2. Any or all of said domains may be wildtype (wt) FVIII domains, or they 50 may differ from the wildtype domains, e.g., as known in the state of the art or deemed appropriate by the skilled person.

[0025] The domains are preferably contained in the protein in that order, i.e., from N-terminus to C-terminus of the protein.

[0026] While parts of the FVIII protein of the invention can be designed as desired by the skilled person, the FVIII preferably maintains a high FVIII biological activity. As shown in the examples, the invention allows generation of a FVIII

protein with a high biological activity, as measured e.g. by the chromogenic activity. Therefore, preferably the FVIII protein according to the invention has a chromogenic activity which is at least comparable to the activity of the wt FVIII protein, i.e., it has at least 50% of the specific chromogenic activity of the wt protein (SEQ ID NO: 1). Preferably, the FVIII protein according to the invention has at least 80%, at least 100 % or more than 100% of the specific chromogenic activity of the wt protein. Preferably, the chromogenic activity also is at least 80%, at least 90%, at least 100% or more than 100% of the chromogenic activity of ReFacto AF® (international non-proprietary name: Moroctocog Alfa), a commercially available B-domain deleted FVIII (Pfizer).

**[0027]** A FVIII protein according to the present invention shall have at least one biological activity or function of a wt FVIII protein, in particular with regard to the function in coagulation. The FVIII protein should be cleavable by thrombin, leading to activation. Preferably, the FVIII protein according to the invention comprises at least one thrombin recognition and/or thrombin cleavage site, wherein said thrombin recognition and/or thrombin cleavage sites may correspond to or substantially correspond to those of wild type FVIII. It is then capable of forming a complex with the activated coagulation Factor IXa and coagulation Factor X, and the light chain is capable of binding to a phospholipid bilayer, e.g., the cell membrane of (activated) platelets.

**[0028]** The biological activity of FVIII can be determined by analyzing the chromogenic, the clotting or the coagulant activity of the protein, as described herein. Typically, the chromogenic activity is taken as a measure of biological activity.

**[0029]** An increased in vivo half-life may be achieved for Factor VIII proteins of the invention that are double chain proteins. Double chain proteins which may form a basis for the FVIII proteins of the invention are known in the art, e.g., wt FVIII or B-domain deleted or truncated versions thereof, e.g., ReFacto AF®.

**[0030]** Moreover, the inventors have found an excellent increase in in vivo half-life with Factor VIII proteins of the invention that are a single chain proteins. Single chain Factor VIII proteins which may form a basis for the FVIII proteins of the invention are known in the art. In general, single chain FVIII proteins do not comprise a functional furin cleavage site and thus, before activation, remain in the circulation as a single chain. Such proteins are also disclosed in EP application No. 19173440 or taught herein.

**[0031]** As a single chain backbone for the inventive protein, a single chain FVIII molecule has been developed, in which several amino acids including the furin cleavage site (positions R1664 - R1667, wherein the signal peptide is also counted) have been deleted. The B domain is deleted to a large extent, wherein an internal fragment (at least NPP) of the B-domain is maintained and an intact Thrombin cleavage site is preceding the internal fragment. This single chain Factor VIII protein (FVIII-sc) has been shown to be more stable than wt Factor VIII. Thus, in a preferred embodiment, the recombinant Factor VIII protein of the invention comprises, in a single chain, a heavy chain portion comprising an A1 and an A2 domain and a light chain portion comprising an A3, C1 and C2 domain of Factor VIII, wherein

a) in said recombinant Factor VIII protein, 894 amino acids corresponding to consecutive amino acids between F761 and P1659 of wild type Factor VIII as defined in SEQ ID NO: 1 are deleted, leading to a first deletion;

b) said recombinant Factor VIII protein comprises, spanning the site of the first deletion, a processing sequence comprising SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site;

c) in said recombinant Factor VIII protein, at least the amino acids corresponding to amino acids R1664 to R1667 of wild type Factor VIII are deleted, leading to a second deletion; and

d) said recombinant Factor VIII protein comprises, C-terminal to the second deletion and N-terminal of the A3 domain, a second thrombin cleavage site.

**[0032]** As defined in a), in the FVIII of the invention, 894 amino acids corresponding to consecutive amino acids between F761 and P1659 of wild type Factor VIII as defined in SEQ ID NO: 1 are deleted in the Factor VIII protein of the invention, leading to a first deletion. In certain embodiments, in particular, starting from a numbering of amino acids in FVIII without deletions or insertions, the term "corresponding to" should be understood to mean "identical to".

**[0033]** For specific amino acids which may be mutated compared to the wt, an amino acid corresponding to the wild type aa is determined by an alignment e.g. using EMBOSS Needle (based on the Needleman-Wunsch algorithm; settings: MATRIX: "BLOSUM62", GAP OPEN: "20", GAP EXTEND:"0.5", END GAP PENALTY: "false", END GAP OPEN: "10", END GAP EXTEND: "0.5").

**[0034]** In order to assess sequence identities of two polypeptides, such an alignment may be performed in a two-step approach: I. A global protein alignment is performed using EMBOSS Needle (settings: MATRIX: "BLOSUM62", GAP OPEN: "20", GAP EXTEND:"0.5", END GAP PENALTY: "false", END GAP OPEN: "10", END GAP EXTEND: "0.5") to identify a particular region having the highest similarity. II. The exact sequence identity is defined by a second alignment using EMBOSS Needle (settings: MATRIX: "BLOSUM62", GAP OPEN: "20", GAP EXTEND:"0.5", END GAP PENALTY:

"false", END GAP OPEN: "10", END GAP EXTEND: "0.5") comparing the fully overlapping polypeptide sequences identified in (I) while excluding non-paired amino acids.

**[0035]** "between" excludes the recited amino acids, e.g., it means that the recited amino acids are maintained. "deletion" or "deleted" does not necessitate that the protein was actually prepared by deleting amino acids previously present in a predecessor molecule, but it merely defines that the amino acids are absent, independent from the preparation of the molecule. For example, the protein can be produced based on nucleic acids prepared by *de novo* synthesis or by genetic engineering techniques.

**[0036]** As defined in b), the recombinant Factor VIII protein comprises, spanning the site of the first deletion, a processing sequence comprising SEQ ID NO: 2 (PRSFSQLNPP) or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site. Accordingly, at least one amino acid of the processing sequence corresponds to an amino acid C-terminal to the deletion and at least one amino acid of the processing sequence corresponds to an amino acid N-terminal to the deletion. The processing sequence comprises SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, i.e., the processing sequence can be longer. In particular, the processing sequence is selected from the group comprising SEQ ID NO: 2, 4, 5, 6, 7 or 8. The inventors have found that a processing sequence of the invention enables a particularly good cleavage by thrombin.

**[0037]** In preferred embodiments, the processing sequence is no longer than SEQ ID NO: 4. The processing sequence may be directly C-terminal to sequences from the a2 domain, e.g., wt a2 domain sequences. The first N-terminal two amino acids of the processing sequence may already belong to the a2 domain. Preferably, the amino acid directly N-terminal to the processing sequence is E.

**[0038]** One amino acid in SEQ ID NO: 2 can be substituted, e.g., to reduce immunogenicity. Optionally, the F, the S C-terminal to the F, the Q or the N are substituted.

**[0039]** The processing sequence may be SEQ ID NO: 4 (PRSFSQLNPPVL) or a sequence having at most one amino acid substitution in said sequence, wherein, optionally, the F, the S C-terminal to the F, the Q or the N are substituted. Moreover, the present inventors have shown that an L at the C-terminus of the processing sequence, as in SEQ ID NO: 4, 5, 6, 7 or 8, endows the FVIII with particularly good activity. One especially preferred example of a single chain FVIII protein, which may form the backbone for the protein of the invention, is shown in the examples in further detail under the name V0 (SEQ ID NO: 16). The processing sequence of the FVIII protein V0, which has been found to be particularly advantageous, consists of SEQ ID NO: 4, which is a specific embodiment of SEQ ID NO: 5-8.

**[0040]** The alternative processing sequences SEQ ID NO: 5 (PRSFSQLNPPVL), SEQ ID NO: 6 (PRSFSQLNPPVL), SEQ ID NO: 7 (PRSFSQLNPPVL) and SEQ ID NO: 8 (PRSFSQLNPPVL) are variants in which X can be any naturally occurring amino acid. Optionally, X is a conservative substitution compared to the corresponding amino acid in SEQ ID NO: 4, i.e. a hydrophobic amino acid is substituted by a hydrophobic amino acid, a hydrophilic amino acid is substituted by a hydrophilic amino acid, an aromatic amino acid by an aromatic amino acid, an acid amino acid by an acid amino acid and a basic amino acid by a basic amino acid.

**[0041]** As defined in c), in the FVIII protein of the invention, the amino acids corresponding to amino acids R1664 to R1667 of wild type Factor VIII are deleted, leading to a second deletion. These amino acid correspond to the furin cleavage recognition site of wt FVIII. Accordingly, the protein is essentially not cleaved by furin. In a composition, at least 80%, optionally, at least 90% or at least 95% of the FVIII protein of the invention are present in a single chain form.

**[0042]** As defined in d), the recombinant Factor VIII protein of the invention comprises, C-terminal to the second deletion and N-terminal of the A3 domain, a second thrombin cleavage site. Accordingly, upon activation, the part of the FVIII protein between the thrombin cleavage site in the processing sequence and the second thrombin cleavage site are excised from the activated FVIII protein.

**[0043]** Further, the invention provides a recombinant Factor VIII protein comprising, in a single chain, a heavy chain portion comprising an A1 and an A2 domain and a light chain portion comprising an A3, C1 and C2 domain of Factor VIII, wherein,

a) said recombinant Factor VIII protein comprises a processing sequence comprising SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site;

b) directly C-terminal to said processing sequence, said Factor VIII protein comprises a heterologous sequence comprising at least one, preferably, two albumin binding domain(s);

c) directly C-terminal to said heterologous sequence, said Factor VIII protein comprises a merging sequence having at least 90% sequence identity to SEQ ID NO: 9 (e.g., SEQ ID NO: 9); and

d) said recombinant Factor VIII protein comprises, C-terminal to SEQ ID NO: 9, a second thrombin cleavage site; and

e) said recombinant Factor VIII protein comprises, C-terminal to the light chain portion, at least one, preferably, two albumin binding domain(s).

5 [0044] Said recombinant FVIII protein may be a FVIII protein as described above. The FVIII protein typically comprises at least one further thrombin cleavage site.

10 [0045] In one embodiment, a FVIII protein of the invention that optionally is a single chain protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa1667 of SEQ ID NO: 1, and a light chain portion having at least 90% sequence identity to aa1668-aa2351 of SEQ ID NO: 1. Optionally, the respective sequence identity aa20-aa1667 of SEQ ID NO: 1 and sequence identity to aa1668-aa2351 of SEQ ID NO: 1 are at least 95%. The respective sequence identity to aa20-aa1667 of SEQ ID NO: 1 and sequence identity to aa1668-aa2351 of SEQ ID NO: 1 may be at least 98%. Optionally, the respective sequence identity to said sequences is at least 99%. The invention also provides a FVIII protein of the invention comprising a heavy chain portion having aa20-aa1667 of SEQ ID NO: 1 and a light chain portion having aa1668-aa2351 of SEQ ID NO: 1.

15 [0046] Several experiments performed by the inventors have been carried out with a single chain FVIII of the invention on the basis of the V0 single chain construct (SEQ ID NO: 16) with at least one albumin binding domain introduced C-terminal to the heavy chain portion and C-terminal to the light chain portion, as described herein. Such proteins have shown advantageous characteristics with regard to expression, stability, in vivo half-life and purification. Accordingly, a preferred FVIII protein of the invention, which may be a single chain protein, comprises a heavy chain portion having at least 90% sequence identity to aa20-aa768 of SEQ ID NO: 16, and a light chain portion having at least 90% sequence identity to aa769-aa1445 of SEQ ID NO: 16. Optionally, the respective sequence identity to aa20-aa768 of SEQ ID NO: 16 and sequence identity to aa769-aa1445 of SEQ ID NO: 16 are at least 95%. The respective sequence identity to aa20-aa768 of SEQ ID NO: 16 and sequence identity to aa769-aa1445 of SEQ ID NO: 16 may be at least 98%. Optionally, the respective sequence identity to said sequences is at least 99%. The invention also provides a FVIII protein of the invention comprising a heavy chain portion having aa20-aa768 of SEQ ID NO: 16 and a light chain portion having aa769-aa1445 of SEQ ID NO: 16.

20 [0047] wt FVIII typically is bound by vWF. vWF shields FVIII from proteolytic degradation and receptor-mediated clearance, e.g. via low-density lipoprotein (LDL) receptor-related protein (LRP1), LDL-receptor (LDLR) and heparan-sulfate proteoglycans (HSPG), within the liver (Lenting et al., 2007. J Thromb Haemost 5:1353-60). However, it has been shown that the half-life of vWF is approx. 15 h, thereby limiting the FVIII:vWF complex half-life to the vWF-related 25 clearance pathway. The inventors found that vWF binding potency of the FVIII protein of the invention may be diminished compared to wt FVIII or ReFacto AF®, which may be explained by sterical hindrance due to albumin binding. For example, FVIII proteins of the invention may have 0%-90%, 10%-80%, 20%-70%, 30%-60%, or 40%-50% of the binding potency of ReFacto AF® to vWF, which can be determined by an assay as described below. Preferably, in the presence of human serum albumin in physiological concentrations, said binding potency is less than 50% of the binding potency of ReFacto AF® to vWF.

30 [0048] vWF binding is mediated in particular by amino acid positions Y1683 and Y1699. To avoid vWF binding, e.g. amino acids corresponding to Y1683 and/or Y1699 of wt FVIII of SEQ ID NO: 1 may be mutated. For example, the amino acid corresponding to Y1683 and/or Y1699 of wt FVIII of SEQ ID NO: 1 may be mutated to a C or F, e.g., Y1699C or Y1699F. In particular, a mutation of the amino acid corresponding to Y1699 to F and a mutation of the amino acid corresponding to Y1683 to F, both mutations together also designated "b mutation" have been confirmed to further decrease binding of vWF to FVIII proteins of the invention. Beside the "b mutation", the inventors have additionally tested an "a mutation" comprising the amino acid substitutions Y737F, Y738F, and Y742F of wt FVIII of SEQ ID NO: 1 and a "c mutation" comprising the amino acid substitutions I2117S and R2169H of wt FVIII of SEQ ID NO: 1. Additionally, the inventors have tested combinations of "a mutation" and "b mutation" and further combinations of "a mutation" and "b mutation" and "c mutation". It was observed, that the "c mutation" negatively influenced the protein expression and functionality. The "b mutation" either alone or in combination with the "a mutation" did not influence the protein expression and functionality, but strongly decreased the binding to vWF. In comparison, the "a mutation" did not decrease the binding to vWF.

35 [0049] Thus, to further decrease vWF binding, the recombinant Factor VIII protein of the invention may have a suitable mutation as described herein, e.g., a "b mutation", i.e., a mutation of the amino acid corresponding to Y1699 to F at position 1699 and a mutation of the amino acid corresponding to Y1683 to F at position 1683 in wt Factor VIII protein of SEQ ID NO: 1. For example, the FVIII protein of the invention may comprise a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least two albumin binding domains (e.g., two) are C-terminal to the heavy chain portion and at least two albumin binding domains (e.g., two) are C-terminal to the light chain portion, wherein the FVIII protein further comprises a b mutation. Such a FVIII protein may further comprise linkers, e.g., a thrombin cleavable linker optionally flanked by a glycine-serine linker, between the albumin binding domains and other parts of the protein and between the albumin binding domains. Alternatively, such a FVIII protein does not comprise linkers. In the context of the invention, "flanked" means that the relevant portions are in a close vicinity,

preferably, with a distance of at most 10, 5 or 2 amino acids positions. Optionally, the relevant portions are immediately adjacent.

5 Albumin binding domains

[0050] The recombinant Factor VIII protein of the invention comprises a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion.

[0051] Different albumin-binding domains (ABDs) may be employed in the context of the invention. ABDs comprise or are protein domains capable of binding albumin, preferably with high affinity, e.g., with at least 50% or at least 80% of the affinity of the ABD2 to albumin. Historically, the ABD typically is a small, three-helical protein domain derived from one of various surface proteins expressed by gram-positive bacteria. For example, domains derived from streptococcal protein G and protein PAB from *Finegoldia magna*, which share a common origin and therefore represent an interesting evolutionary system, have been thoroughly studied structurally and functionally. Their albumin-binding sites have been mapped and these domains form the basis for a wide range of protein engineering approaches. By substitution-mutation they have been engineered to achieve a broader specificity, an increased stability or an improved binding affinity, respectively.

[0052] For example, albumin binding domains disclosed by Nilvebrant et al. (2013, Comput Struct Biotechnol J. 6: e201303009), Johansson et al. (2001, JBC 277: 8114-8120), Jacobs et al. (2015, Protein Engineering, Design and Selection 28 (10): 385-393), WO 91/01743 A1, WO 2009/016043 A2, WO 2010/054699 A1, WO 2012/004384 A2, WO 2014/048977 A1 or WO 2015/091957 A1 may be used.

[0053] Preferably, the albumin binding domain comprises a sequence according to SEQ ID NO: 44: LAX<sub>3</sub>AKX<sub>6</sub>X<sub>7</sub>ANX<sub>10</sub>ELDX<sub>14</sub>YGVSDFYKRLIX<sub>26</sub>KAKTVEGVEALKX<sub>39</sub>X<sub>40</sub>ILX<sub>43</sub>X<sub>44</sub>LP wherein independently of each other

25 X<sub>3</sub> is selected from E, S, Q and C, preferably, E;  
 X<sub>6</sub> is selected from E, S, C and V, preferably, E;  
 X<sub>7</sub> is selected from A, S and L, preferably, A;  
 X<sub>10</sub> is selected from A, S and R, preferably, A;  
 30 X<sub>14</sub> is selected from A, S, C and K, preferably, S;  
 X<sub>26</sub> is selected from D, E and N, preferably, D;  
 X<sub>39</sub> is selected from D, E and L, preferably, D;  
 X<sub>40</sub> is selected from A, E and H, preferably, A;  
 X<sub>43</sub> is selected from A and K, preferably, A;  
 35 X<sub>44</sub> is selected from A, S and E, preferably, A;  
 L in position 45 is present or absent, preferably, present; and  
 P in position 46 is present or absent, preferably, present.

[0054] Alternatively, the albumin binding domain may comprise an amino acid sequence which has at least 95 % 40 identity to the sequence of SEQ ID NO: 44.

[0055] The inventors have achieved good results using an albumin binding domain designated ABD1 (SEQ ID NO: 45). It is preferred to use the sequence of ABD2 (SEQ ID NO: 46) that has been deimmunized for the human immune system, i.e., adapted to avoid immune responses in humans. If not otherwise mentioned, said albumin binding domain is used in the experiments shown herein. ABD2 may be encoded by a nucleic acid of SEQ ID NO: 57, which is codon-45 optimized for expression in human cells.

[0056] While it is possible to use different albumin binding domains at different locations in the FVIII protein, typically, all albumin binding domains in the FVIII protein will have the same sequence, preferably ABD2. Alternatively, it is possible to use different albumin binding domains at different locations within the FVIII protein in order to obtain multivalent albumin binding.

[0057] For example, in the FVIII protein of the invention, one albumin binding domain may be C-terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion. Alternatively, in one of the two selected positions, there may be one albumin binding domain, and two, three, four or more albumin binding domains in the other. For example, one albumin binding domain may be C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion, or one albumin binding domain may be C-terminal to the heavy chain portion and three albumin binding domains C-terminal to the light chain portion, or one albumin binding domain may be C-terminal to the heavy chain portion and four albumin binding domains C-terminal to the light chain portion.

[0058] In a FVIII protein of the invention, two albumin binding domains may be C-terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion, or three albumin binding domains may be C-

terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion, or four albumin binding domains may be C-terminal to the heavy chain portion and one albumin binding domain C-terminal to the light chain portion.

**[0059]** Preferably, the number of albumin binding domains in each of the two positions is the same. It has also been shown to be advantageous if the FVIII protein of the invention comprises at least four albumin binding domains. The inventors have found a still better increase in the half-life for Factor VIII protein of the invention comprising at least two albumin binding domains C-terminal to the heavy chain portion and at least two albumin binding domains C-terminal to the light chain portion, preferably, two albumin binding domains C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion.

**[0060]** The invention also provides Factor VIII proteins of the invention with two albumin binding domains C-terminal to the heavy chain portion and three albumin binding domains C-terminal to the light chain portion, or with two albumin binding domains C-terminal to the heavy chain portion and four albumin binding domains C-terminal to the light chain portion, or with three albumin binding domains C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion, or with four albumin binding domains C-terminal to the heavy chain portion and two albumin binding domains C-terminal to the light chain portion. Optionally, there is an even number of albumin-binding domains both C-terminal to the heavy chain and C-terminal to the light chain.

#### Linker

**[0061]** While the inventors have shown that linkers are not principally required for activity and stability of the FVIII proteins of the invention, to increase accessibility of all domains of the FVIII of the invention, in particular, access to albumin in blood, linkers were introduced into some FVIII proteins of the invention. The inventors have shown that the linkers, in particular, inclusion of at least glycine-serine linker sections, further improve expression and function. In particular, in addition to access to albumin, access for thrombin also appears to be improved. Accordingly, preferably, albumin binding domains may be separated from the heavy chain portion and/or the light chain portion and/or other albumin-binding domains by a linker, wherein, optionally, albumin-binding domains are separated from the heavy chain portion and the light chain portion and (if directly adjacent otherwise) other albumin-binding domains by a linker. It is also possible that albumin-binding domains are separated from the heavy chain portion and the light chain portion and (if directly adjacent otherwise) other albumin-binding domains by a linker, except that there is no linker N-terminal to the light chain, because the a3 domain contains a thrombin cleavage site.

**[0062]** In one preferred embodiment, in the recombinant Factor VIII protein of the invention, the linker comprises a thrombin-cleavable linker section. Optionally, a thrombin cleavable linker has the sequence of SEQ ID NO: 39 (abbr. L). Further thrombin cleavable sites are known in the art, e.g., disclosed in Gallwitz et al. (2012, PLoS ONE 7 (2): e31756). Thrombin cleavable linkers may thus also comprise any of these cleavable sites. Thrombin-cleavable linkers have the advantage that in generation of the active protein, i.e., after activation by thrombin, the linkers may be cleaved and, consequently, the albumin binding domains may be removed from the active protein.

**[0063]** Alternatively, in the recombinant Factor VIII protein of the invention, uncleavable glycine-serine linker sections may be used to introduce flexible, sterical distance between motifs to avoid structural influences. Thus, optionally, the linker comprises a glycine-serine linker section that optionally has the sequence of SEQ ID NO: 40 (abbr. G1, preferred) or SEQ ID NO: 41 (abbr. G2). The linker G1 may, e.g., be encoded by SEQ ID NO: 58. The linker G2 may, e.g., be encoded by SEQ ID NO: 59.

**[0064]** In preferred embodiments, different linker sections are combined. E.g. uncleavable linker sections were used to flank a central thrombin cleavable linker section to maintain thrombin accessibility of the thrombin cleavable linker section. Thus, in some embodiments, the linker comprises a thrombin cleavable linker section flanked on each side by a glycine-serine linker section, wherein said combined linker optionally has the sequence of SEQ ID NO: 42 or SEQ ID NO: 43, preferably, SEQ ID NO: 42.

**[0065]** The polynucleotide sequence for all linkers preferably is codon-optimized for human use. Exemplary codon-optimized sequences are provided herein in the context of FVIII proteins of the invention.

#### Specific FVIII proteins of the invention

**[0066]** All FVIII proteins of the invention demonstrated good in vitro functionality, wherein the FVIII proteins showed reduced vWF binding in correlation to increasing numbers of albumin binding domains. vWF has a major impact on the half-life of FVIII. It was found that shielding FVIII from vWF by albumin positively influences the half-life of the FVIII protein. A broad distribution of albumin binding domains with one position between heavy chain and light chain and one position at the C-terminus of the protein was shown to enhance the shielding of FVIII from vWF.

**[0067]** Preferably, the recombinant Factor VIII protein the invention comprises one albumin binding domain between the heavy chain portion and the light chain portion and one albumin binding domain C-terminal to the light chain portion,

wherein the sequence has at least 70%, optionally, at least 80%, at least 90%, at least 95%, at least 99% or 100% sequence identity sequence identity to SEQ ID NO: 47. Preferably, said protein is a single chain protein. The single chain protein having SEQ ID NO: 47 is designated ADLCD\_SC. It was shown to have an in vivo half-life increased by a factor of about 1.5 compared to ReFacto AF®.

5 [0068] In an especially preferred embodiment, the recombinant Factor VIII protein of the invention comprises at least two albumin binding domains between the heavy chain portion and the light chain portion and at least two albumin binding domain C-terminal to the light chain portion, wherein the protein has at least 80% sequence identity, optionally, at least 90%, at least 95% or at least 99% sequence identity to any of SEQ ID NO: 48, 49, 51. Preferably, the recombinant Factor VIII protein has at least 80% sequence identity, optionally, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 48. Preferably, said protein is a single chain protein.

10 [0069] The recombinant Factor VIII protein may also have at least 80% sequence identity, optionally, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 49. Preferably, said protein is a single chain protein.

15 [0070] The recombinant Factor VIII protein may also have at least 80% sequence identity, optionally, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 51. Preferably, said protein is a single chain protein.

20 [0071] For example, the invention provides a recombinant FVIII protein having SEQ ID NO: 48 (AD2CD2\_SC), SEQ ID NO: 49 (AD2CD2wol\_SC), or SEQ ID NO: 51 (AbD2CD2\_SC). Such FVIII proteins have been shown to have a particularly extended in vivo half-life, e.g., for AD2CD2\_SC, an in vivo half-life extended by a factor of 2.5 has been found in hemophilia A mice and a half-life extension of factor 4 has been found in albumin-deficient, transgenic neonatal Fc-receptor mice (see Examples). For AbD2CD2\_SC, an in vivo half-life extended by a factor of 2.2 has been found.

25 An increase in half-life can be analyzed on the level of FVIII antigen or on the level of activity, e.g., chromogenic activity, or both. Preferably, it is analyzed on the level of chromogenic activity.

30 [0072] The invention thus provides FVIII proteins of the invention, wherein the in vivo half-life of the recombinant Factor VIII protein is prolonged (i.e. increased) by a factor of at least 1.2, preferably, by a factor of at least 1.5, optionally, at least 2 or at least 2.5 in comparison to a recombinant Factor VIII protein of SEQ ID NO: 28 (ReFacto AF®). While the increase in in vivo half-life may be analyzed in model systems, e.g., mice, rats or dogs, such as in hemophilia A mice or albumin-deficient Tg32 mice having a knock-out of murine albumin and expressing human FcRn a-chain instead of the murine one (B6.Cg-Alb<sup>em12Mvw</sup> Fcgr<sup>tm1Dcr</sup> Tg(FCGRT)32Dcr/MvwJ), the observed increase in in vivo half-life may be underestimated, because human albumin has a longer half-life than e.g. murine albumin, and it is expected that an increase seen in a murine model will be still more pronounced in humans.

35 [0073] A fusion partner may be employed to extend the in vivo plasma half-life of the FVIII protein of the invention. In one embodiment, the recombinant Factor VIII protein of the invention is a fusion protein with at least one further heterologous fusion partner in addition to the albumin binding moiety, preferably with a further fusion partner extending the in vivo plasma half-life of the FVIII protein. The fusion partner may e.g. be selected from the group comprising an Fc region, albumin, PAS polypeptides, HAP polypeptides, the C-terminal peptide of the beta subunit of chorionic gonadotropin, and combinations thereof. The FVIII protein may alternatively or additionally be covalently linked to non-protein fusion partners such as albumin-binding small molecules (e.g., dabigatran), PEG (polyethylenglycol) and/or HES (hydroxyethyl starch). PAS polypeptides or PAS sequences are polypeptides comprising an amino acid sequence comprising mainly alanine and serine residues or comprising mainly alanine, proline and serine residues, the PAS sequences forming a random coil conformation under physiological conditions, as defined in WO 2015/023894. HAP polypeptides or sequences are homo-amino acid polymer (HAP), comprising e.g., repetitive sequences of glycine or glycine and serine, as defined in WO 2015/023894. Potential fusions, fusion partners and combinations thereof are described in more detail e.g., in WO 2015/023894.

40 [0074] Optionally, for certain therapeutic applications, the recombinant FVIII protein may be fused to an Fc region. A fusion to an Fc region may be used to extend the half-life and reduce immunogenicity.

45 [0075] Optionally, said heterologous fusion partner may be inserted directly N-terminal or directly C-terminal to one of the albumin binding domains, e.g., C-terminal to the heavy chain, and/or C-terminal to the C2-domain, or C-terminal to the albumin binding domain(s) C-terminal to the heavy chain or C-terminal to the albumin binding domain(s) C-terminal to the heavy chain. These locations have been found by the inventors to be advantageous for fusion, while maintaining good biological activity of the FVIII protein. Optionally, the fusion protein further comprises at least one linker.

50 [0076] The protein may further be glycosylated and/or sulfated. Preferably, post-translational modifications such as glycosylation and/or sulfation of the protein occur in a human cell. A particularly suitable profile of post-translational modifications can be achieved using human cell lines for production, e.g. CAP cells, in particular CAP-T cells or CAP-Go cells (WO 2001/36615; WO 2007/056994; WO 2010/094280; WO 2016/110302). CAP cells, available from Cevac Pharmaceuticals GmbH (Cologne, Germany), originate from human amniocytes as they were isolated trans-abdominally during routine amniocentesis. Obtained amniocytes were transformed with adenoviral functions (E1A, E1B, and pIX functions) and subsequently adapted to growth in suspension in serum-free medium.

55 [0077] During post-translational modification, the FVIII protein of the invention may be sulfated, e.g., on one, two, three, four, five or six tyrosines in the acidic regions a1, a2 and a3.

**[0078]** Optionally, the recombinant Factor VIII protein of the invention is a de-immunized protein, i.e., the protein has a reduced immunogenicity as compared to wt FVIII in hemophilia patients. For example, certain mutations, preferably, substitutions, have been introduced to avoid the presence of epitopes which can be presented on human HLA molecules, preferably, common human HLA molecules.

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#### Nucleic acids and host cells

**[0079]** The invention also provides a nucleic acid encoding a recombinant Factor VIII protein of the invention. Said nucleic acid may be an expression vector, e.g., suitable for expression of said recombinant Factor VIII protein in a mammalian cell, such as a human cell, such as a CAP cell.

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**[0080]** The nucleic acid preferably encodes the FVIII with an N-terminal signal sequence, e.g., the 19 aa signal sequence of SEQ ID NO: 1. Preferred nucleic acids of the invention encode a recombinant FVIII protein having SEQ ID NO: 47 (ADLCD\_SC), SEQ ID NO: 48 (AD2CD2\_SC), SEQ ID NO: 49 (AD2CD2woL\_SC), SEQ ID NO: 50 (AD2CD2woLG\_SC) or SEQ ID NO: 51 (AbD2CD2\_SC). They may be SEQ ID NO: 52-56. The nucleic acids of the invention may be DNA molecules or RNA molecules. The nucleic acids may be optimized for expression in the respective host cell, e.g., in a human cell, e.g., a CAP cell.

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**[0081]** The expression vector comprises the sequence encoding the FVIII protein, preferably, in codon-optimized form, under the functional control of a suitable promoter, which may be a constitutive or an inducible promoter. The promoter may be a promoter not associated with expression of FVIII in nature, e.g., EF-1 alpha or a heterologous promoter, e.g., CMV or SV40. It may further comprise pro- and/or eukaryotic selection markers, such as ampicillin resistance and dihydrofolate reductase (dhfr), and origins of replication, e.g., an SV40 origin and/or a pBR322 origin. "codon-optimized" means optimized for expression in the host cell, preferably, for expression in a human host cell.

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**[0082]** In certain embodiments, the nucleic acid may be a vector suitable for gene therapy, e.g., for gene therapy of a human patient. Vectors suitable for gene therapy are known in the art, e.g., virus-based vectors e.g., based on adenovirus or adeno-associated virus (AAV) or based on retrovirus, such as lentiviral vectors etc., or non virus-based vectors such as but not limited to small plasmids and minicircles or transposon-based vectors. An AAV-based vector of the invention may e.g., be packaged in AAV particles for gene therapy of Hemophilia A patients.

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**[0083]** The invention also provides a host cell comprising a nucleic acid of the invention. The host cell may be a bacterial cell, a plant cell, a fungal cell, a yeast cell or an animal cell. Preferably, the host cell is an animal cell, in particular, a mammalian cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said cell. The host cell preferably is a human cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said human cell. The cell may be transiently or stably transfected with the nucleic acid of the invention. The cell may be a cell line, a primary cell or a stem cell. For production of the protein, the cell typically is a cell line such as a HEK cell, such as a HEK-293 cell, a CHO cell, a BHK cell, a human embryonic retinal cell such as Crucell's Per.C6 or a human amniocyte cell such as CAP. For treatment of human patients with the protein, the host cell preferably is a human cell, e.g., a HEK293 cell line or a CAP cell line (e.g. a CAP-T cell or a CAP-Go cell). The inventors have found that in a CAP cell line, a particularly high single chain content of FVIII protein of the invention is produced. Among the CAP cells, CAP-T cells are preferred for transient expression, while CAP-Go cells may be used for creation of stable cell lines conveying an advantageous glycosylation profile to the FVIII molecule.

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**[0084]** The cell may be an autologous cell of a Hemophilia A patient, in particular, a human Hemophilia A patient, suitable for producing FVIII in the patient after transfection and reintroduction into the patient's body. The cell may be a stem cell, e.g., a hematopoietic stem cell, but preferably it is not an embryonic stem cell, in particular when the patient is a human. The cell may also be hepatocyte, a liver sinusoidal endothelial cell or a thrombocyte.

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**[0085]** Cell lines expressing the protein of the invention may also be used in a method of preparing the protein of the invention, comprising cultivating said cells under conditions suitable for expression of the FVIII protein and purifying said protein, e.g., using a plurality of methods known to the skilled person, such as described herein. Such purification methods may comprise standard harvesting procedures for cell removal, e.g. centrifugation, followed by chromatography steps, e.g. affinity chromatography, and methods for exchanging the FVIII proteins into a suitable buffer. The invention thus also provides a method for preparing a Factor VIII protein, comprising culturing the host cell of the invention under conditions suitable for expression of the Factor VIII protein and isolating the Factor VIII protein, wherein the method optionally comprises formulating the Factor VIII protein as a pharmaceutical composition.

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#### Pharmaceutical compositions

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**[0086]** The invention provides a pharmaceutical composition comprising the recombinant Factor VIII protein of the invention, the nucleic acid of the invention or the host cell of the invention. Such pharmaceutical compositions may comprise suitable excipients or carriers, e.g., a buffer, a stabilizing agent, a bulking agent, a preservative, another (e.g., recombinant) protein or combinations thereof. In the context of the invention, if not explicitly stated otherwise, "a" is

understood to mean one or more.

[0087] A suitable buffer for formulation of the protein of the invention may e.g. contain 205 mM NaCl, 5.3 mM CaCl<sub>2</sub>, 6.7 mM L-Histidine, 1.3 % Sucrose and 0.013 % Tween 20 in distilled water and have a pH of 7.0 (FVIII formulation buffer). Said buffer is used in the experiments described herein if not otherwise stated. Formulations of FVIII may be 5 sterile, e.g., sterile filtered, in particular for in vivo use.

[0088] Optionally, the pharmaceutical composition of the invention comprising FVIII protein further comprises albumin, preferably, for a human patient, human serum albumin. Albumin may, e.g., be in a concentration of 0.1-10% w/w, such as 1-5% human serum albumin (w/w). Albumin may bind to the ABD of the FVIII proteins of the invention either before administration, or after administration to a human subject.

[0089] The pharmaceutical composition may be formulated as desired appropriate by the skilled person, e.g., for intravenous (i.v.) or subcutaneous application, intraperitoneal or intramuscular application. Generally, it is for administration as slow i.v. push bolus injection. Continuous infusion is indicated e.g., for patients requiring admission for severe bleeds or surgical procedures. Oral application, which may contribute to tolerance induction, is also possible, e.g., after 10 expression in plants. The pharmaceutical composition may be for slow release.

[0090] Pharmaceutical compositions comprising FVIII can be lyophilized.

[0091] Due to the increase in half-life in vivo, the pharmaceutical compositions of the invention may be administered at longer intervals than previous FVIII compositions. For example, they may be for use in administration every 5 to 14 days, preferably, every 7 to 10 days.

[0092] Dosages and treatment schemes may be chosen as appropriate, e.g., for prophylaxis of bleeding or with 20 intermittent, on-demand therapy for bleeding events. Decisions on dosing may be made by the physician. Dosing depends on the patent, e.g., weight, FVIII status, severity of disease etc. For example, the FVIII of the invention may be administered in dosages of 0.5 to 250 IU/kg body weight every 0.5 to 14 or every 6-7 days intravenously depending on the severity of the disease, typically, 0.5 to 200 IU/kg body weight.

[0093] The invention also provides a pharmaceutical composition comprising the FVIII protein of the invention in 25 combination with an immunosuppressive agent (e.g., methylprednisolone, prednisolone, dexamethasone, cyclophosphamide, rituximab, and/or cyclosporin), and/or it may be for administration at substantially the same time (e.g. within five minutes to within 12 hours) with such an agent. The invention thus also provides a kit comprising, in addition to a FVIII protein of the invention, optionally, combined with albumin, an immunosuppressive agent, e.g., an immunosuppressive agent selected from the group comprising methylprednisolone, prednisolone, dexamethasone, cyclophosphamide, rituximab, and/or cyclosporin.

[0094] The pharmaceutical composition, e.g., comprising the protein of the invention, may be for use in treating a patient in need thereof, in particular, a Hemophilia A patient, e.g., a patient with acquired hemophilia involving an autoimmune response to FVIII or a congenital Hemophilia A patient. Mammals such as mice or dogs may be treated with the pharmaceutical composition of the invention, but the patient typically is a human patient.

[0095] The pharmaceutical composition of the invention may also be used for treatment of a patient previously already treated with a recombinant and/or plasmatic Factor VIII protein. In a patient who has an antibody including an inhibitory antibody response to a recombinant and/or plasmatic Factor VIII protein, the pharmaceutical compositions may, e.g., be used for immune tolerance induction (ITI) treatment. The compositions of the invention may thus also be used for 30 rescue ITI. The pharmaceutical compositions may also be advantageously used in a patient who has had an antibody response including an inhibitory antibody response to a recombinant and/or plasmatic Factor VIII protein, e.g., who has been treated by ITI. The pharmaceutical compositions may also be advantageously used in a patient who has had an antibody response including an inhibitory antibody response to a recombinant and/or plasmatic Factor VIII protein, who has not been treated by ITI.

[0096] The invention also provides a vial comprising the pharmaceutical composition of the invention, e.g., a syringe. 45 The syringe may be a pre-filled syringe, e.g., a ready-to-use syringe.

[0097] The inventors contemplate that, in the context of gene therapy, due to the presence of the albumin binding domains, long-term expression and folding of the FVIII protein of the invention may be improved compared to expression of conventional FVIII.

[0098] All publications cited herein are fully incorporated herewith. The invention is further illustrated by the following 50 embodiments, figures and examples, which are not to be understood as limiting the scope of the invention.

### Embodiments

[0099] In summary, in a first embodiment, the invention provides a recombinant Factor VIII protein comprising a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion, wherein, if the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion.

[0100] In a second embodiment, the recombinant Factor VIII protein of the first embodiment (embodiment 1) is a single chain protein.

[0101] In a third embodiment, the recombinant Factor VIII protein of embodiment 1 is a double chain protein.

[0102] In a fourth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion.

[0103] In a fifth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion.

In a sixth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and three albumin binding domains are C-terminal to the light chain portion. In a seventh embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, one albumin binding domain is C-terminal to the heavy chain portion and four albumin binding domains are C-terminal to the light chain portion.

[0104] In an eighth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, two albumin binding domains are C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion. In a ninth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, three albumin binding domains are C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion.

[0105] In a tenth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, four albumin binding domains are C-terminal to the heavy chain portion and one albumin binding domain is C-terminal to the light chain portion.

[0106] In an eleventh embodiment, in the recombinant Factor VIII protein of any of embodiments 1-3, at least two albumin binding domains are C-terminal to the heavy chain portion and at least two albumin binding domains are C-terminal to the light chain portion, preferably, two albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion. In a twelfth embodiment, in the recombinant Factor VIII protein of embodiment 11, two albumin binding domains are C-terminal to the heavy chain portion and three albumin binding domains are C-terminal to the light chain portion. In a thirteenth embodiment, in the recombinant Factor VIII protein of embodiment 11, two albumin binding domains are C-terminal to the heavy chain portion and four albumin binding domains are C-terminal to the light chain portion. In a fourteenth embodiment, in the recombinant Factor VIII protein of embodiment 11, three albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion. In a fifteenth embodiment, in the recombinant Factor VIII protein of embodiment 11, four albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion.

[0107] In a sixteenth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-15, albumin-binding domains are separated from the heavy chain portion and/or the light chain portion and/or other albumin-binding domains by a linker, wherein, preferably, albumin-binding domains are separated from the heavy chain portion and the light chain portion and other albumin-binding domains by a linker.

[0108] In a seventeenth embodiment, in the recombinant Factor VIII protein of embodiment 16, the linker comprises a Thrombin-cleavable linker section that optionally has the sequence of SEQ ID NO: 39.

[0109] In an eighteenth embodiment, in the recombinant Factor VIII protein of any of embodiments 16 or 17, the linker comprises a glycine-serine linker section that optionally has the sequence of SEQ ID NO: 40 or SEQ ID NO: 41.

[0110] In a nineteenth embodiment, in the recombinant Factor VIII protein of any of embodiments 16-18, said linker is a combination of different linker sections, e.g. the linker comprises a Thrombin-cleavable linker section flanked on each side by a glycine-serine linker section, wherein said linker optionally has the sequence of SEQ ID NO: 42 or SEQ ID NO: 43.

[0111] In a twentieth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-19, the albumin binding domain comprises a sequence according to SEQ ID NO: 44.

[0112] In a twenty-first embodiment, in the recombinant Factor VIII protein of embodiment 20, the albumin binding domain comprises a sequence according to SEQ ID NO: 46.

[0113] In a twenty-second embodiment, in the recombinant Factor VIII protein of any of embodiments 1-21, the heavy chain portion comprises the domains A1 and A2, and optionally comprises the domains A1-a1-A2-a2 or A1-a1-A2-a2-B.

[0114] In a twenty-third embodiment, in the recombinant Factor VIII protein of any of embodiments 1-22, the light chain portion comprises the domains A3 and C1 and C2, and optionally comprises the domains a3-A3-C1-C2.

[0115] In a twenty-fourth embodiment, in the recombinant Factor VIII protein of any of embodiments 1-23, the B-domain of the Factor VIII protein is at least partly deleted.

[0116] In a twenty-fifth embodiment, the recombinant Factor VIII protein of any of embodiments 1-24 comprises, in a single chain, a heavy chain portion comprising an A1 and an A2 domain and a light chain portion comprising an A3, C1 and C2 domain of Factor VIII, wherein

55 a) in said recombinant Factor VIII protein, 894 amino acids corresponding to consecutive amino acids between F761 and P1659 of wild type Factor VIII as defined in SEQ ID NO: 1 are deleted, leading to a first deletion;

b) said recombinant Factor VIII protein comprises, spanning the site of the first deletion, a processing sequence comprising SEQ ID NO: 2 or a sequence having at most one amino acid substitution in SEQ ID NO: 2, wherein said processing sequence comprises a first thrombin cleavage site;

5 c) in said recombinant Factor VIII protein, at least the amino acids corresponding to amino acids R1664 to R1667 of wild type Factor VIII are deleted, leading to a second deletion; and

d) said recombinant Factor VIII protein comprises, C-terminal to the second deletion and N-terminal of the A3 domain, a second thrombin cleavage site.

10 [0116] In a twenty-sixth embodiment, the recombinant Factor VIII protein of any of embodiments 1-25 that optionally is a single chain protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa768 of SEQ ID NO: 16 and a light chain portion having at least 90% sequence identity to aa769-aa1445 of SEQ ID NO: 16, wherein said sequence identities preferably are at least 95%, at least 98% or 100%.

15 [0117] In a twenty-seventh embodiment, the recombinant Factor VIII protein of any of embodiments 1-26 that optionally is a single chain protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa1667 of SEQ ID NO: 1 and a light chain portion having at least 90% sequence identity to aa1668-aa2351 of SEQ ID NO: 1, wherein said sequence identities optionally are at least 95%, at least 98% or 100%. In a twenty-eighth embodiment, the recombinant Factor VIII protein of any of embodiments 1-4 and 16-27, comprises one albumin binding domain between the heavy 20 chain portion and the light chain portion and one albumin binding domain C-terminal to the light chain portion, wherein the sequence has at least 70% sequence identity to SEQ ID NO: 47.

25 [0118] In a twenty-ninth embodiment, the recombinant Factor VIII protein of any of embodiments 11-28 is a single chain protein comprising at least two albumin binding domains between the heavy chain portion and the light chain portion and at least two albumin binding domain C-terminal to the light chain portion, wherein the protein has at least 80% sequence identity, optionally, at least 90% sequence identity, at least 95% sequence identity or at least 98% sequence identity to any of SEQ ID NO: 48, 49 or 51. In a thirtieth embodiment, the recombinant Factor VIII protein of embodiment 29 has at least 80% sequence identity to SEQ ID NO: 48, e.g., it has SEQ ID NO: 48 or SEQ ID NO: 51.

30 [0119] In a thirty-first embodiment, the recombinant Factor VIII protein of any one of embodiments 1-30 has a "b mutation", i.e., a mutation of the amino acid corresponding to Y1699 to F at position 1699 and a mutation of the amino acid corresponding to Y1683 to F at position 1683 in wt Factor VIII protein of SEQ ID NO: 1.

[0120] In a thirty-second embodiment, the in vivo half-life of the recombinant Factor VIII protein of any one of embodiments 1-31 in a human subject is prolonged by a factor of at least 1.2, preferably, by a factor of at least 1.5, optionally, at least 2 or at least 2.5 in comparison to a recombinant Factor VIII protein of SEQ ID NO: 28.

35 [0121] In a thirty-third embodiment, the recombinant Factor VIII protein of any one of embodiments 1-32 is a fusion protein with at least one fusion partner selected from the group consisting of an Fc region, albumin, PAS polypeptides, HAP polypeptides, the C-terminal peptide of the beta subunit of chorionic gonadotropin, polyethylenglycol, and hydroxethyl starch.

[0122] In a thirty-fourth embodiment, the recombinant Factor VIII protein of any one of embodiments 1-33 is a de-immunized protein.

40 [0123] In a thirty-fifth embodiment, the invention provides a nucleic acid encoding a recombinant Factor VIII protein of any one of embodiments 1-34. In a thirty-sixth embodiment, the nucleic acid of embodiment 35 is an expression vector, preferably, suitable for expression of said recombinant Factor VIII protein in a mammalian cell, preferably, in a human cell, such as a CAP cell.

45 [0124] In a thirty-seventh embodiment, the invention provides a host cell comprising a nucleic acid of any of embodiments 35 or 36. In a thirty-eighth embodiment, the host cell of embodiment 37 is a mammalian cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said cell, preferably, a human cell selected from the group comprising a Hek293 cell or a CAP cell (e.g., CAP-T cell or CAP-Go cell).

50 [0125] In a thirty-ninth embodiment, the invention provides a method of preparing a recombinant Factor VIII protein, comprising culturing the host cell of embodiments 37 or 38 under conditions suitable for expression of the Factor VIII protein and isolating the recombinant Factor VIII protein, wherein the method optionally comprises formulating the Factor VIII protein as a pharmaceutical composition.

55 [0126] In a fortieth embodiment, the invention provides a pharmaceutical composition comprising the recombinant Factor VIII protein of any of embodiments 1-34, the nucleic acid of any of embodiments 35-36 or the host cell of any of embodiments 37-38. In a forty-first embodiment, the pharmaceutical composition of embodiment 40 further comprises a biologically acceptable carrier such as water or a buffer, optionally, at a physiologic pH, preferably, FVIII formulation buffer, and/or pharmaceutically acceptable excipients. In a forty-second embodiment, the pharmaceutical composition of any of embodiments 40 or 41 further comprises albumin, e.g., 0.1-5%, such as 1% human serum albumin.

[0127] In a forty-third embodiment, the invention provides a pharmaceutical composition of any of embodiments 40-42

or a kit further comprising an immunosuppressive agent, e.g., an immunosuppressive agent selected from the group comprising methylprednisolone, prednisolone, dexamethasone, cyclophosphamide, rituximab, and/or cyclosporin.

[0128] The invention further provides, as a forty-fourth embodiment, a pharmaceutical composition of any of embodiments 40-43 for use in treatment of hemophilia A, wherein, optionally, the treatment is immune tolerance induction (ITI).

5 In a forty-fifth embodiment, the pharmaceutical composition of any of embodiments 40-44 is for use in treating a patient with Hemophilia A selected from the group comprising a patient not previously treated with any Factor VIII protein, a patient previously treated with a Factor VIII protein, a patient who has an antibody response including an inhibitory antibody response to a Factor VIII protein, and a patient who has had an antibody response including an inhibitory antibody response to a Factor VIII protein who has been treated by ITI, or who has not been treated by ITI.

10 [0129] In a forty-sixth embodiment, the pharmaceutical composition of any of embodiments 40-45 is for administration every 5 to 14 days, preferably, every 7 to 10 days.

[0130] In a forty-seventh embodiment, the invention provides a vial, e.g., a prefilled or ready-to use syringe, comprising the pharmaceutical composition of any of embodiments 40-46.

15 [0131] In a forty-eighth embodiment, the invention provides a method of treatment, comprising administering an effective amount of the pharmaceutical composition of any of embodiments 40-46 to a patient in need thereof, e.g., a patient with hemophilia A, which may be selected from the patient groups defined herein.

SEQ ID NO: 1	wt human FVIII
SEQ ID NO: 2	processing sequence in preferred single chain constructs
20 SEQ ID NO: 4	processing sequence in V0
SEQ ID NO: 5	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 6	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 7	variant processing sequence, X can be varied for de-immunization
25 SEQ ID NO: 8	variant processing sequence, X can be varied for de-immunization
SEQ ID NO: 9	merging sequence, e.g., in V0
SEQ ID NO: 16	single chain FVIII V0
SEQ ID NO: 28	6rs-REF
30 SEQ ID NO: 39	Thrombin-cleavable linker
SEQ ID NO: 40	glycine-serine linker G1, G in position 14 is present or absent
SEQ ID NO: 41	glycine-serine linker G2
SEQ ID NO: 42	Thrombin-cleavable linker flanked on each side by a glycine-serine linker, strictly repetitive
SEQ ID NO: 43	Thrombin-cleavable linker flanked on each side by a glycine-serine linker, non-repetitive
35 SEQ ID NO: 44	ABD consensus sequence, see above
SEQ ID NO: 45	ABD1
SEQ ID NO: 46	ABD2
SEQ ID NO: 47	ADLCLD_SC aa
SEQ ID NO: 48	AD2CD2_SC aa
40 SEQ ID NO: 49	AD2CD2woL_SC aa
SEQ ID NO: 50	AD2CD2woLG_SC aa
SEQ ID NO: 51	AbD2CD2_SC aa
SEQ ID NO: 52	ADLCLD_SC na
45 SEQ ID NO: 53	AD2CD2_SC na
SEQ ID NO: 54	AD2CD2woL_SC na
SEQ ID NO: 55	AD2CD2woLG_SC na
SEQ ID NO: 56	AbD2CD2_SC na
50 SEQ ID NO: 57	optimized DNA sequence encoding SEQ ID NO: 46
SEQ ID NO: 58	exemplary DNA encoding Glycine-serine linker G1 of SEQ ID NO: 40
SEQ ID NO: 59	exemplary DNA encoding Glycine-serine linker G2 of SEQ ID NO: 41

### Figure Legends

55 [0132]

Fig. 1 shows the human albumin binding of ADLCLD\_SC, a FVIII protein of the invention comprising two albumin-

binding domains in comparison to FVIII 6rs-Ref, a protein having the ReFacto AF sequence. Both FVIII proteins were tested in the presents and absence of HSA via the albumin binding capacity assay as described.

5 Fig. 2 shows the von-Willebrand factor (vWF) binding capacity of different FVIII-albumin-binding-domain fusion proteins in relation of ReFacto AF. All FVIII molecules were tested for their vWF binding in either the presence or absence of human albumin. The more albumin-binding domains were incorporated into FVIII, the lower was the binding to vWF in general. The presence of human albumin dramatically decreased the binding to vWF.

10 Fig. 3 Comparison of unpurified FVIII-ABD fusion variants and FVIII controls for their in vitro functionality. Cell culture supernatants of CAP-T cells expressing the double chain FVIII molecule 6rs-REF, the single chain FVIII molecule AC\_SC, and the FVIII-ABD fusion molecules AD2CD2\_SC, AD2CD2woLG\_SC, AD2CD2wL\_SC, ACD4woLG\_SC, and ACL(GD)<sub>4</sub>\_SC were analyzed for chromogenic FVIII activity (A), FVIII clotting activity induced by Actin FSL (B) and FVIII antigen levels indicating total FVIII protein amount (C). Specific chromogenic activity was calculated as chromogenic FVIII activity to FVIII antigen ratio displayed in % (D). Specific clotting activity was calculated as FVIII clotting activity to FVIII antigen ratio displayed in % (E). n=2.

15 Fig. 4 Western blot analysis of unpurified FVIII-ABD fusion variants and FVIII control proteins for assessing structural properties. Cell culture supernatants of CAP-T cells expressing the double chain FVIII molecule 6rs-REF, the single chain FVIII molecule AC\_SC, and the FVIII-ABD fusion molecules AD2CD2\_SC, AD2CD2woLG\_SC, AD2CD2wL\_SC, and ACD4woLG\_SC were separated by non-reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequent blotting onto a PVDF membrane was performed. A purified Sheep anti-Human Factor VIII primary antibody and CF680-conjugated donkey anti-sheep IgG (H&L) antibody was used for detection. For size determination, Precision Plus All Blue was applied as marker.

20 25 Fig. 5 demonstrates the in vivo pharmacokinetics of AD2CD2\_SC compared to ReFacto AF after a single injection of 200 U/kg FVIII (with 1% human albumin) into mice having a knock-out for murine albumin and expressing the a-chain of human instead of murine neonatal Fc-receptor. Determined FVIII antigen values were normalized and are shown in percent over time.

30 Fig. 6 demonstrates the in vivo pharmacokinetics of AD2CD2\_SC compared to ReFacto AF after a single injection of 30 U FVIII Antigen/kg formulated with either 1 or 10% human albumin into Göttingen minipigs. Plasma samples were withdrawn and FVIII antigen levels were measured by ELISA. Mean FVIII antigen levels are shown in U/ml over time in hours. n=3 minipigs per group.

35 Fig. 7 shows the total bleeding time (first column of each group, left Y-axis) and the total blood loss (second column of each group, right Y-axis) after tail transection of hemophilia A mice which were administered 20 h earlier with either Vehicle Control, ReFacto AF, Elocate, AD2CD2\_SC or ADLCLD\_SC. Non-hemophilia C57BL/6NCrl mice were treated with 0.9% NaCl and used as control.

## 40 Examples

### 1. Evaluation of Factor VIII proteins with additions of albumin-binding domains for the development of a new hemophilia A therapeutic

#### 45 Material and methods

##### Preparation of constructs

50 [0133] Experiments were performed to find and develop a suitable backbone for integration of the albumin-binding domains. The experiments were done on the basis of a B-domain deleted version of FVIII and single chain variants of FVIII. The basic double chain construct was a codon-optimized sequence of ReFacto AF® (Pfizer), wherein for simplifying cloning, 6 restriction sites were added through silent mutations, but some of these restriction sites were again excluded due to codon-optimization. The basic double chain sequence is 6rs-REF (SEQ ID NO: 28). The basic single chain construct used was V0 (SEQ ID NO: 16, EP19173440)

55 [0134] Firstly, the ABD protein sequence (Affibody AB, Solna, Sweden) was taken as a basis for design of the DNA sequence. If not mentioned otherwise, the ABD2 sequence was used. Moreover, codon optimized linkers were developed, which are partly cleavable by thrombin. If not otherwise stated, the glycine-serine linker was G1 and the thrombin-cleavable linker was L. Table 1 below demonstrates structures of fusion proteins with albumin-binding domains (ABD)

for single chain molecules.

[0135] For the constructs encoding the FVIII of the invention and comparative constructs also analysed in this context, either the complete FVIII sequence or DNA regions carrying approx. 700-1200 bp from the FVIII a2 domain to the A3 domain were synthesized. The synthesized DNA was codon-optimized for the total target gene. The a2 to A3 DNA fragments were 5' terminally flanked by an EcoRV restriction site, and 3' terminally flanked by an EcoRI restriction site, and these restriction sites were also present in the basic FVIII sequence used. For C-terminal fusion to the light chain, DNA fragments carrying approx. 1500-2100 bp were synthesized also in a codon-optimized form. Such DNA fragments were 5' terminally flanked by an EcoRI restriction site within the A3 domain, and 3' terminally flanked by an NotI restriction site. Restriction of the DNA inserts and the FVIII backbone plasmid allowed for targeted ligation and generation of FVIII single chain plasmids. Completely synthesized FVIII DNA was 5' terminally flanked by a HindIII restriction site, and 3' terminally flanked by a NotI restriction site.

[0136] By transformation of *E.coli* K12 with said plasmids, expansion of transformed bacteria under ampicillin selection and plasmid preparation, large amounts of the plasmids could be prepared. Genetic engineering work was carried out by Thermo Fisher Scientific after design with VectorNTI Software (Thermo Fisher Scientific, Massachusetts, USA).

#### Cultivation of CAP-T cells

[0137] For analyzing the candidates for new recombinant FVIII molecules, the constructs, integrated in expression vectors, were transiently and stably expressed in human cell lines. The preferred cell lines are Hek293 and CAP cells, both of which originate from human amniocytes. Because of higher yields of active FVIII molecules CAP cells, in particular, CAP-T cells were chosen as the preferred expression system for transient transfection and CAP-Go cells for stable expression.

[0138] Transient transfection was performed with nucleofection programs. The supernatants were screened for FVIII activity and antigen. Purification of the recombinant proteins from CAP cells was done, including FVIII affinity chromatography.

[0139] In detail, CAP-T cells (Cevec Pharmaceuticals, Köln, Germany) were cultured in PEM medium supplemented with 4 mM GlutaMAX (Thermo Fisher Scientific, 35050038) and 5 µg/ml blasticidin (Thermo Fisher Scientific, R21001; complete PEM medium). In order to thaw the cells, the required amount of frozen vials were transferred to a 37 °C water bath. After thawing, each vial was transferred to 10 ml of chilled, complete PEM medium. The cell suspension was centrifuged at 150 x g for 5 minutes. During this washing step the dimethyl sulfoxide (DMSO) used for cryopreservation was removed. The pellet was resuspended in 15 ml warm, complete PEM medium and transferred to a 125 ml shaker flask. The cells were incubated at 37 °C in a humidified incubator with an atmosphere containing 5 % CO<sub>2</sub>. The flasks were set on a shaking platform, rotating at 185 rpm with an orbit of 50 mm. Subculturing of the cells was performed every 3 to 4 days. The fresh culture was set to 0.5x10<sup>6</sup> cells/ml by transferring the required amount of cultured cell suspension to a new flask and adding complete PEM medium. In the case that the transferred cell suspension would exceed 20% of the total volume, the suspension was centrifuged at 150 x g for 5 minutes and the pellet was resuspended in fresh complete PEM medium. The volume of cell suspension per shaking flask was 20% of the total flask volume.

[0140] A minimum of three subcultures were performed after thawing before transfection experiments were performed.

#### Protein expression in CAP-T cells by transient transfection

[0141] The CAP-T cells were transfected using the 4D-Nucleofector™ (Lonza, Basel, Switzerland). For each transfection 10x10<sup>6</sup> CAP-T cells were centrifuged at 150 x g for 5 minutes in 15 ml conical tubes. The cells were resuspended in 95 µl supplemented SE Buffer, taking into account the volume of the pellet and the volume of the plasmid solution. Afterwards, 5 µg of the respective plasmid were added to the cell suspension followed by gentle mixing. The solution was transferred to 100 µl Nucleocuvettes. The used transfection program was ED-100. After the transfection, the cells from one Nucleocuvette were transferred to 125 ml shaker flasks, containing 12.5 ml complete PEM medium. The cells were cultivated for 4 days as described above. At day 4 the cells were harvested by centrifugation at 150 x g for 5 minutes. Larger protein amounts could be produced by combining 12.5 ml approaches as described above.

[0142] Supernatants were screened for FVIII activity and antigen directly after harvest.

[0143] The recombinant Factor VIII protein was further analyzed. FVIII activity was measured by chromogenic activity assay and clotting activity FSL assay. The antigen was estimated by FVIII antigen ELISA. As a further assay for biological activity, the cleavage of the recombinant proteins by thrombin was analyzed. Moreover, chain distribution and appearance was tested by Western Blots. Further, vWF-binding and albumin binding were tested.

#### FVIII activity - chromogenic activity assay

[0144] The activity of FVIII was determined by a chromogenic assay. In this two-step assay, FIXa and FVIIIa activate

FX in the first step. In the second step, the activated FX hydrolyses a chromogenic substrate, resulting in a color change, which can be measured at 405 nm. Due to the fact that calcium and phospholipids are present in optimal amounts and an excess of FIXa and FX is available, the activation rate of FX is only dependent on the amount of active FVIII in the sample.

5 [0145] The reagents for this chromogenic FVIII activity assay were taken from the Coatest® SP FVIII Kit. The kit contained phospholipids, calcium chloride (CaCl<sub>2</sub>), trace amounts of thrombin, the substrate S-2765, a mixture of FIXa and FX and the thrombin inhibitor I-2581. The inhibitor was added, in order to prevent hydrolysis of the substrate by thrombin, which was built during the reaction. All dilutions were performed in distilled water or Tris-BSA (TBSA) Buffer, containing 25 mM Tris, 150 mM sodium chloride (NaCl) and 1 % Bovine serum albumin (BSA), set to pH 7.4. Each sample was diluted at least 1:2 with FVIII-depleted plasma. Further dilutions were performed using the TBSA Buffer.

10 [0146] The assay was performed using the BCS XP (Siemens Healthcare, Erlangen, Germany), a fully automated hemostasis analyzer. All reagents including water, TBSA Buffer and the samples were inserted into the analyzer. For each sample the analyzer mixed 34 µl calcium chloride, 20 µl TBSA Buffer, 10 µl sample, 40 µl water, 11 µl phospholipids and 56 µl FIXa-FX-mixture. This mixture was incubated for 300 seconds. Afterwards, 50 µl of S-2765 + I-2581 were 15 added to the reaction. Upon addition of the substrate, the absorption at 405 nm was measured for 200 seconds.

15 [0147] In order to calculate the amount of active FVIII, the software of the analyzer evaluated the slope of the measured kinetic between 30 seconds and 190 seconds after starting the reaction. This result was correlated to a calibration curve, generated with a biological reference preparation (BRP) of FVIII. The activity of the BRP is indicated in IU/ml. However, 20 IU/ml can be assumed equivalent to U/ml. The results were indicated as "% of normal". These results were converted to U/ml, as 100 % of normal FVIII activity are equivalent to 1 U FVIII activity per ml.

#### Clotting Activity FSL

25 [0148] In addition to the two-stage chromogenic assay (see above), a one-stage clotting assay was also performed in order to determine the amount of active FVIII. During this assay, FVIII-depleted plasma, CaCl<sub>2</sub>, the activator Actin FSL and the FVIII-containing sample are mixed in one step. The activator leads to the generation of FXIa, which activates FIX. FVIIIa, FIXa and FX built the tenase complex and FX becomes activated. Further activation of prothrombin and fibrinogen finally leads to the formation of a fibrin clot. The time needed to form the clot, the activated partial thromboplastin time (aPTT), is measured. The aPTT varies, depending on the amount of FVIII.

30 [0149] The clotting assay was performed using the BCS XP. TBSA Buffer, FVIII-depleted plasma, Actin FSL, CaCl<sub>2</sub> and the sample were inserted into the analyzer. The sample was diluted at least 1:2 with FVIII-depleted plasma. Further dilutions were performed using the TBSA Buffer. For each sample the analyzer mixed 45 µl TBSA Buffer, 5 µl sample, 50 µl FVIII-depleted plasma and 50 µl Actin FSL. The reaction was started by the addition of 50 µl CaCl<sub>2</sub>. The analyzer measured the time needed for clot formation.

35 [0150] In order to calculate the amount of active FVIII, the software of the analyzer evaluated a baseline extinction at 405 nm at the beginning of the reaction. All of the following extinction values, within a time of 200 seconds, were analysed regarding their difference to the baseline extinction. The first time point exceeding a defined threshold was determined as the clotting time. This result was correlated to a calibration curve, generated with a BRP of FVIII.

#### FVIII antigen ELISA

40 [0151] The amount of FVIII antigen was determined using the Aserachrom® VIII:Ag ELISA (Diagnostica Stago, Asnières sur Seine Cedex, France). In this sandwich ELISA, the applied FVIII is bound by mouse monoclonal anti-human FVIII F(ab')<sub>2</sub> fragments, which are coated to the plate by the manufacturer. The detection of the bound FVIII occurs via mouse monoclonal anti-human FVIII antibodies, which are coupled to a peroxidase. In the case that FVIII is present, the peroxidase-coupled antibody binds to FVIII and can be detected by the addition of a tetramethylbenzidine (TMB) solution. TMB turns from a clear to a blue-green solution upon reaction with peroxidase. After a short time, this reaction is stopped by the addition of sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), which turns the solution yellow. The amount of bound FVIII correlates with the intensity of the yellow color, which can be measured at 450 nm. The final amounts of FVIII are calculated using a calibration curve generated by the measurement of at least five serial dilutions of a calibrator with a known antigen concentration.

45 [0152] The supplied calibrator and control were reconstituted with 500 µl of distilled water, 30 minutes before starting the ELISA. After this incubation time, the calibrator was diluted 1:10 in the supplied phosphate buffer. This represented the starting concentration. The calibrator was further serially diluted 1:2 up to a dilution of 1:64. As the concentration of the calibrator contained approximately 1 U/ml FVIII, depending on the batch, the starting concentration was equivalent to 0.1 U/ml FVIII whereas the last dilution contained approximately 0.0016 U/ml FVIII. The control was diluted 1:10 and 50 1:20 with the phosphate buffer. All samples were diluted with the phosphate buffer, depending on their previously determined activity (see above) with the aim to be in the middle of the calibration curve. After the dilution of FVIII samples,

control and calibrator, 200  $\mu$ l of each solution were applied per well in duplicates. In addition to that, two wells were filled with 200  $\mu$ l of phosphate buffer as a blank control. The plate was incubated for 2 hours at room temperature covered with a film. During this time, the peroxidase-coupled anti-human FVIII antibodies were reconstituted with 8 ml phosphate buffer and incubated 30 minutes at room temperature. After the antigen immobilization, the wells were washed five times with the supplied washing solution, which was previously diluted 1:20 with distilled water. Immediately after the washing, 200  $\mu$ l of the peroxidase-coupled anti-human FVIII antibodies were added to each well and incubated for 2 hours at room temperature covered by a film. Afterwards, the plate was washed five times as before. In order to reveal the amount of bound FVIII, 200  $\mu$ l of TMB solution were added to each well and incubated for exactly 5 minutes at room temperature. This reaction was stopped by the addition of 50  $\mu$ l 1 M  $H_2SO_4$  to each well. After an incubation time of 15 minutes at room temperature, the absorbance of each well was measured at 450 nm using the POLARstar Omega plate reader (BMG LABTECH, Ortenberg, Germany).

**[0153]** The results of the ELISA were calculated using the MARS software (BMG Labtech). In a first step, all wells were blank corrected and the mean of the duplicates was calculated. Afterwards, a 4-parameter fit was applied, in order to calculate the concentrations from the calibration curve. According to this calibration curve the amount of FVIII antigen in each well was determined. In the last step, the values were corrected by the dilution factor, resulting in the FVIII antigen amount of each sample.

#### Albumin binding capacity assay

**[0154]** 20% human serum albumin (HSA) was diluted 1:4000 in PBS. 96-well ELISA plates were filled with 100  $\mu$ l/well with diluted HSA solution and coated during a 2 h incubation at 37°C and 400 rpm on a thermoshaker. ELISA plates were washed 3-times with 300  $\mu$ l/well washing buffer. Standard control and FVIII samples either with or without Albumin pre-incubation were diluted with Tris/NaCl pH 7.4 to a concentration of 0.5 U/ml chromogenic activity and 100  $\mu$ l/well were added as 7-step 1:2 serial dilution. Incubation was performed for 1 h at 37°C covered on a thermoshaker. In the meantime, FIXa and FX were resolved together in 10 ml aqua dest., substrate (S-2765 and I-2581) was solved in 12 ml aqua dest.. After FVIII incubation, plates were washed again 3 times with 300  $\mu$ l/well washing buffer. Phospholipides and the FIXa/FX solution were mixed 1:5 and subsequently 50  $\mu$ l/well of this solution were added and incubated for 5 min at 37°C. Without any washing step 25  $\mu$ l  $CaCl_2$  was added to each well, followed by 5 min incubation at 37°C. Finally, 50  $\mu$ l/well substrate were added and detection of activated FX-mediated substrate turnover was performed at 405 nm for 25 cycles followed by end point measurement using an ELISA reader.

#### vWF binding capacity assay

**[0155]** 1 U/ml of each FVIII molecule was either pre-incubated or not with 40 mg/ml albumin for 30 min at RT to promote ABD-albumin binding and an assay for determining the vWF binding capacity was performed as follows: Plasma purified vWF (Biotest AG) was diluted with 0.9% NaCl solution to a concentration of 0.1 U/ml. Coating onto 96-well ELISA plates was done by transferring 100  $\mu$ l of this solution to each well followed by an 2 h incubation at 37°C and 400 rpm. The wells were washed 3 times with 300  $\mu$ l of washing buffer (8 mM sodium phosphate, 2 mM potassium phosphate, 0.14M NaCl, 10 mM KCl, 0.05% Tween-20, pH 7.4). FVIII standard (commercial rFVIII without vWF) and samples were pre-diluted with dilution buffer (25 mM Tris, 150 mM NaCl, pH 7.4) to a concentration of 0.25 U/ml according to chromogenic activity and transferred as a 7-step, serial 1:2 dilution into each plate well (100  $\mu$ l/well). Incubation was carried out for 1 h at 37°C and 400 rpm. In the meantime, FIXa and FX were resolved together in 10 ml aqua dest., substrate (S-2765 and I-2581) was solved in 12 ml aqua dest.. After FVIII incubation, plates were washed again 3 times with 300  $\mu$ l/well washing buffer. Phospholipides and the FIXa/FX solution were mixed 1:5 and subsequently 50  $\mu$ l/well of this solution were added and incubated for 5 min at 37°C. Without any washing step 25  $\mu$ l  $CaCl_2$  was added to each well, followed by 5 min incubation at 37°C. Finally, 50  $\mu$ l/well substrate were added and detection of activated FX-mediated substrate turnover was performed at 405 nm for 25 cycles followed by end point measurement using an ELISA reader.

#### Western Blot

#### Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

**[0156]** Cell supernatants, cell lysates or purified material of FVIII variants were appropriately diluted with 1x NuPAGE LDS Sample Buffer (4x, Thermo Fisher Scientific, NP0007) and further diluted 1:2 with reducing sample buffer. Reducing sample buffer was produced by combining 2.5 parts of NuPAGE LDS Sample Buffer with 1 part of NuPAGE Sample Reducing Agent (10x, Thermo Fisher Scientific, NP0004). 20  $\mu$ l of each sample were mixed with 20  $\mu$ l of reducing sample buffer in a 1.5 ml vial and heated for 10 min at 70°C using a thermoshaker (Eppendorf). A NuPAGE 4-12% Bis-

5 Tris Protein Gel (Thermo Fisher Scientific) was inserted into the XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific) and inner and outer chambers were filled with 1x NuPAGE MOPS SDS Running buffer (Thermo Fisher Scientific, NP0001). 500  $\mu$ l of NuPAGE Antioxidant (Thermo Fisher Scientific) was added to the inner chamber. 10  $\mu$ l of the each prepared sample and 4  $\mu$ l of Precision Plus Protein All Blue Standard (Bio-Rad, 161-0373) diluted 1/10 in 1x LDS Sample Buffer were loaded onto the gel. The sample separation was achieved by running the gel at a constant voltage of 200 V for 50-60 min.

#### Non-reducing SDS-PAGE

10 [0157] 20  $\mu$ l of cell supernatants, cell lysates or purified material of FVIII variants were, either pre-diluted or not, appropriately diluted with 10  $\mu$ l of NuPAGE LDS Sample Buffer (4x, Thermo Fisher Scientific, NP0007) and 10  $\mu$ l aqua dest.. Samples were heated for 10 min at 70°C using a thermoshaker (Eppendorf). A NuPAGE™ 3-8% Tris-Acetate Protein Gel (Thermo Fisher Scientific) was inserted into the XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific) and inner and outer chambers were filled with 1x NuPAGE™ Tris-Acetate SDS Running Buffer (Thermo Fisher Scientific, LA0041). 10  $\mu$ l of the each prepared sample and 4  $\mu$ l of Precision Plus Protein All Blue Standard (Bio-Rad, 161-0373) diluted 1/10 in 1x LDS Sample Buffer were loaded onto the gel. The sample separation was achieved by running the gel at a constant voltage of 150 V for 55-70 min.

#### Western Blotting and Detection

20 [0158] To investigate the separated proteins by immunofluorescence detection, they were transferred onto an Odyssey nitrocellulose membrane (Li-Cor) or an Amersham Hybond Low Fluorescence 0.2  $\mu$ m polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Sciences) by using the XCell II blot module (Thermo Fisher Scientific) for semi-wet protein transfer. The PVDF membrane was activated in methanol and then applied to the SDS gel, whereas the nitrocellulose membrane was directly applied to the SDS gel. The system was filled with NuPAGE transfer buffer (20X, Thermo Fisher Scientific) according to the manufacturer's instructions. Protein blotting was performed for 1 h at 30 V. After protein transfer, the membrane was blocked over night at 4°C in Odyssey blocking buffer (Li-Cor). Afterwards the membrane was incubated for 1 h at room temperature simultaneously with either a rabbit anti-coagulation factor VIII monoclonal antibody (Sino Biological, 13909-R226, 1:1000) and a mouse anti-human factor VIII monoclonal antibody (Merck, MAB038, 1:2500) or with 0.0004  $\mu$ g/ $\mu$ l sheep anti-human factor VIII:C polyclonal antibody (Cedarlane, CL20035AP, 1.5000), each diluted in Odyssey Blocking buffer containing 0.05% Tween 20. After incubation, the membrane was washed 4-times for 5 min in 0.1% PBST. For detection of FVIII heavy and light chain the membrane was incubated with 0.067  $\mu$ g/ml IRDye 800CW donkey anti-mouse (Li-Cor, 926-32212, 1:15000) and 0.067  $\mu$ g/ml IRDye 680RD donkey anti-rabbit (Li-Cor, 926-68073, 1:15000) diluted in Odyssey blocking buffer containing 0.05% Tween 20 for 1 h at room temperature. Alternatively, the CF680 donkey anti-sheep IgG (H&L) antibody (Biotium, 20062-1) was used in a 1:5000 dilution in Odyssey blocking buffer for binding the respective primary antibody. Finally, the membrane was washed 4 times for 5 min in 0.1% PBST, 2 times for 5 min in PBS and rinsed in water. The membrane was visualized using the Licor Odyssey Imager.

#### Pharmacodynamic studies

40 [0159] Coagulation factors were administered by a single intravenous tail vein injection into female haemophilia A mice with doses of 200 U/kg body weight or respective amounts of a control solution. After 4 or 20 h post dosing, tail vein transection bleeding assay was performed as follows: The animals were anaesthetised with 5% isoflurane in 30% O2 and 70% N2O, and immediately placed in prone position on a heating pad at +37°C. Tail vein transection was performed as described by Johansen et al., 2016. Haemophilia 22(4):625-631.

45 [0160] Bleeding was monitored for 60 min and bleeding time was determined using a stop clock. Primary bleeding time was noted until first bleeding cessation. After the primary bleeding, the tail was put into a new centrifuge tube filled with pre-warmed saline. If the mouse was not bleeding at 15, 30 and 45 min post injury, the tail was lifted out of the saline and the wound was challenged by gently wiping it twice with a saline wetted gauze swab in the distal direction. Immediately after the challenge, the tail was re-submerged into the saline. The cumulative bleeding time of all following bleeds constitute the secondary bleeding time. The total bleeding time is defined as the sum of the primary and all secondary bleeding times.

50 [0161] Following the determination of bleeding time, the tubes were centrifuged at 4140 g at room temperature for 3 minutes. Apart from 1 mL, the supernatant was removed. The cell pellet was resuspended and hemoglobin content was determined by using a method similar to that described by Elm et al. (2012).

## Results and Discussion

[0162] Generation and prescreening of several different FVIII-ABD fusion proteins covering FVIII double chain and single chain constructs was very promising in initial experiments and similar in both single chain and double chain backbones.

[0163] The formation of single chain FVIII molecule was increased compared to double chain forms when ABD was fused in between heavy chain and light chain (data not shown).

[0164] The FVIII proteins further developed and produced shown herein are listed in Table 1.

10 **Table 1 Structure of relevant variants of FVIII with ABD fusion.** A= FVIII A1+a1+A2+a2+truncated B domain, C= FVIII a3 (optionally truncated) +A3+C1+C2 domains, L= Thrombin cleavable linker, G= flexible glycine-serine linker 1 (G1), D= ABD2,

Construct name	Structure	Protein of the invention?
AD2CD2_SC	ALDGLGDLCLDGLGD_SC	Yes
ADLCLD_SC	ADLCLD_SC	Yes
AbD2CD2_SC	ALDGLGDLCLDGLGD_SC + Y1699F + Y1683F	Yes
AD2CD_SC	ALDGLGDLCLD_SC	Yes
ACLD_SC	ACLD_SC	No
ADLC_SC	ADLC_SC	No
AD2C_SC	AD2C_SC	No
ACLDGLGD_SC	ACLDGLGD_SC	No
AD2CD2woLG_SC	ADDCD2_SC	Yes
AD2CD2woL_SC	AGDGDGCGDGD_SC	Yes
ACD4woLG_SC	ACDDDD_SC	No
ACL(GD)4_SC	ACLDGDGDGD_SC	No

35 [0165] Six single chain FVIII-ABD fusion molecules were generated *in silico* and respective DNA constructs were tested for their expression in either HEK293 or CAP-T cells (cf. Table 3). As all of those FVIII-ABD variants were expressed, secreted and functional, based on results of the chromogenic FVIII activity measurement, all molecules were produced in midi-scale CAP-T cell culture and successfully purified in larger amounts as needed for further characterizations and PK (pharmacokinetic) analysis.

40 **Table 3 FVIII-ABD fusion proteins analyzed in supernatants of transfected HEK293 or CAP-T cells.** (n/a: not available)

Construct	Chromogenic activity [U/mL]	Clotting activity [U/mL]	FVIII: Ag [U/mL]	Specific chromogenic activity [%]	Expression Cell line
ADLC_SC	0,98	0,9	0,47	209	HEK293
ACLD_SC	1,26	0,79	0,72	175	HEK293
ADLCLD_SC	1	0,45	n/a	n/a	HEK293
AD2CD2_SC	1,45	0,35	0,71	204	CAP-T
AD2CD_SC	1,3	0,5	0,48	271	CAP-T
AD2C_SC	2,0	1,0	n/a	n/a	CAP-T
ADLC_SC	0,9	n/a	n/a	n/a	CAP-T
ADLCLD_SC	1,9	n/a	n/a	n/a	CAP-T

**[0166]** All six purified FVIII-ABD fusion variants were extensively characterized by several methods including determination of FVIII antigen and chromogenic activity, Actin FSL clotting, heavy and light chain detection by western blotting (WB), thrombin-cleavage analysis and binding to vWF and albumin. Table 4 gives an overview of produced FVIII-ABD variants in terms of chromogenic and clotting activity as well as antigen levels in the final solutions. Measurement of these values indicated that FVIII-ABD fusion proteins are still capable of their biological function: Bridging factor IXa and factor X leading thereby to the activation of the latter one. Comparison of the specific chromogenic activity (chromogenic activity / antigen \*100) demonstrates that ADLC\_SC and ReFacto AF® are similar (109% vs 104%). However, the specific chromogenic activities of all other FVIII-ABDs are much better, ranging from 130% to 206%.

**[0167]** Interestingly, the results indicate that increasing numbers of ABD motifs within one FVIII molecule decrease the clotting activity and also the capability of vWF binding. The decrease in clotting activity may be caused by the setup of the assay, which is strictly time-dependent. This may not mirror *in vivo* clotting activity.

**Table 4 Measurements of chromogenic FVIII activity, FSL clotting activity, and antigen levels. The indicated specific activity was calculated by the ratio of chromogenic activity and antigen.**

FVIII-ABD variant	chromogenic activity [U/ml]	FSL clotting [U/ml]	Antigen [U/ml]	Specific activity [%]
ADLC_SC	147	101	135	109
ACLD_SC	150	100	101	149
ADLCLD_SC	128	80	62	206
AD2CD_SC	120	61	92	130
AD2CD2_SC	164	42	96	171
AD2C_SC	112	72	78	144
ReFacto AF® (Control)	253	242	243	104

**[0168]** Western blot was used to observe the heavy and light chain patterns of the different FVIII-ABD fusion molecules in comparison to ReFacto AF® (data not shown). The analysis demonstrated that, in contrast to ReFacto AF®, the FVIII-ABD variants were mostly expressed as single chain molecules.

**[0169]** Activation of FVIII-ABD variants was investigated by direct incubation with thrombin at 37°C for 8 min and subsequent provision for reducing SDS-PAGE followed by western blotting. Band patterns of thrombin-activated or untreated FVIII-ABD molecules show that all FVIII-ABD molecules were activated by Thrombin in a comparable manner as ReFacto AF® (data not shown).

**[0170]** Albumin binding of the ADLCLD\_SC variant was tested by an assay in comparison to FVIII 6rs-Ref, demonstrating the capability of albumin binding (Fig 1). An excess of unbound soluble albumin inhibited the binding to plate-bound albumin.

**[0171]** The influence of ABD and linker modifications on the binding between FVIII and vWF were investigated in two settings: 1. directly, without the presence of albumin; 2. after a 30 min pre-incubation with physiological concentrations of albumin promoting the ABD-albumin binding. As demonstrated in Fig. 2, the vWF binding of FVIII-ABD fusion proteins directly decreases by an increasing number of ABD motifs. However, only one ABD motif per FVIII does not have an influence on the vWF binding in the absence of albumin, independent from its position within the molecule. When FVIII-ABDs were pre-incubated with albumin, a decreased vWF binding was observed for all FVIII-ABD variants. This reduction of vWF binding was higher the more ABD domains were incorporated into FVIII.

**[0172]** To investigate the impact of linkers on the production and functionality of FVIII-ABD variants, one preferred variant, AD2CD2\_SC, was also produced (I) without any linkers between FVIII and ABD-Domains (AD2CD2woLG\_SC) and (II) with G1 linkers but without thrombin-cleavable L linkers (AD2CD2wol\_SC). These variants were compared to the double chain FVIII 6rs-Ref (ReFacto amino acid sequence), single chain FVIII backbone AC\_SC and two FVIII-ABD variants having four C-terminal ABD domains either without any linkers (ACD4woLG\_SC) or with one thrombin-cleavable linker followed by four ABD domains separated by G1 linkers (ACL(GD)4\_SC).

**[0173]** Respective plasmids encoding the different FVIII variants were nucleofected into CAP-T cells and 4-day cell culture supernatants were tested for chromogenic FVIII activity, FVIII clotting activity and FVIII antigen levels according to the above-described methods. As shown in Fig. 3, AD2CD2woLG\_SC, ACD4woLG\_SC, and ACL(GD)4\_SC were expressed in only low amounts and chromogenic activity was strongly decreased. AD2CD2woLG\_SC was not expressed in high amounts, but had some specific chromogenic activity. No FVIII clotting activity could be detected for any of these variants. In comparison to all other controls, AD2CD2\_SC and AD2CD2wol\_SC demonstrated good FVIII antigen levels and great FVIII chromogenic and clotting activities, resulting in superior specific chromogenic activity values of approx.

200% or higher. AD2CD2\_SC demonstrate an especially high specific clotting activity.

[0174] A western blot analysis based on a non-reduced SDS-PAGE separation of these variants is demonstrated in Fig. 4. Except for FVIII 6rs-Ref, all other variants are mainly present as single chain FVIII molecules. However, AD2CD2woLG\_SC and AD2CD2woL\_SC tend to form multimers or aggregates, which are not observed for the AD2CD2\_SC variant.

### 3. Pharmacokinetic experiments

[0175] Purification of FVIII ABD variants was performed for in vivo experiments, based on supernatants of transfected CAP-T cells by strong anion exchange chromatography and affinity chromatography.

[0176] To investigate the half-life prolongation effect of ABD motifs introduced into the FVIII molecules, two pharmacokinetic (PK) studies were performed in hemophilia A mice. 12 mice per test item were used, 2 or 3 for each time point. All FVIII-ABD molecules were administered in a single dose of 200 U/kg body weight (6 ml/kg) into the tail vein by a single intravenous tail vein injection into female haemophilia A mice (B6, 129S4-F8<tm1Kaz>/J). Plasma samples taken 0.5, 4, 8, 12, and 20h (and 24h) post injection were analyzed regarding FVIII chromogenic activity and antigen levels in citrate plasma which was subsequently extracted by centrifugation. Plasma samples were stored at -80°C and analyzed for FVIII antigen and chromogenic activity. ReFacto AF® was tested as control beside the FVIII-ABD variants.

[0177] Results are shown in Table 5.

Table 5 Calculated t<sub>1/2</sub> of FVIII-ABD variants. \*AD2C\_SC data were highly variable. t<sub>1/2</sub> is specified in hours

FVIII-ABD variant	Antigen: t <sub>1/2</sub>	Chromogenic activity: t <sub>1/2</sub>
Study 1		
ACLD_SC	4.8	4.3
AD2CD_SC	4.3	5.0
AD2CD2_SC	14.5	9.4
ReFacto® (control)	5.7	4.1
Study 2		
ADLC_SC	7.8	6.8
ADLCLD_SC	10.4	10.5
AD2C_SC*	13.0	6.1
ReFacto® (control)	7.0	6.8

[0178] Thus, by pharmacokinetic studies in hemophilia A mice, preferred FVIII proteins of the invention were identified which show a half-life prolonged up to 2.5x (e.g., ADLCLD\_SC - about 1.5x; AD2CD2\_SC - about 2.5x). Pharmacokinetics of AbD2CD2\_SC were tested in a separate study and were similar to AD2CD2\_SC.

[0179] It is noted that the hemophilia A mouse model may even underestimate half-life extension due to the discrepancy of murine and human albumin (murine albumin only has a half-life of about two days). Nevertheless, the observed relative extended half-life of the FVIII proteins of the invention already allows a potential reduction of intravenous FVIII injection in hemophilia patients from 2 - 3 days to a once weekly dosing.

[0180] Moreover, a pharmacokinetic proof of concept study in albumin-deficient Tg32 mice having a knock-out of murine albumin and expressing human FcRn a-chain instead of the murine one (B6.Cg-Alb<sup>em12Mvw</sup> Fcgr<sup>tm1Dcr</sup> Tg(FC-GRT)32Dcr/MvwJ) was performed. This mouse model (Alb-/mFcRn-/hFcRn<sup>+</sup>), compared to hemophilia A mice, reveals a situation closer to humans as injected human albumin has a half-life of approx. 20 days which is similar to the half-life in humans. Intravenous FVIII injection (AD2CD2\_SC; ReFacto AF®) was done with 200 U/kg (based on chromogenic activity) plus 1% human albumin.

[0181] The results, shown in Fig. 5 demonstrated a half-life extension of AD2CD2\_SC in comparison to ReFacto AF® of about 4x, allowing a potential reduction in patients of i.v. FVIII injection from 2 - 3 days to a 8 - 12 days dosing.

[0182] In addition, a pharmacokinetic study was performed in Göttingen Minipigs. Three animals per group were injected with 30 U FVIII antigen/kg body weight with either (I) ReFacto AF + 1% human serum Albumin (HSA), (II) ReFacto AF + 10% HSA, (III) AD2CD2\_SC + 1% HSA or (IV) AD2CD2\_SC + 10% HSA via the ear vein. Blood samples were taken predose, 4, 12, 36, 48, and 120 h post administration and citrate plasma was isolated immediately by centrifugation. Bioanalytical sample measurement was performed by FVIII antigen ELISA, which is specific for human

FVIII and does not detect any porcine FVIII. Evaluation by non-compartment analysis (Figure 6) obtained half-lives for (I) ReFacto AF + 1% HSA of 7.1 h, (II) ReFacto AF + 10% HSA of 6.4 h, (III) AD2CD2\_SC + 1% HSA of 18.6 h, and (IV) AD2CD2\_SC + 10% HSA of 20.7 h. Thus, an half-life extension of approx. 3-fold was observed for AD2CD2\_SC compared to ReFacto AF in this model.

5 [0183] Moreover, pharmacodynamics studies have been performed. Hemophilia A mice (Jackson No. B6; 129S4-F8<tm1 Kaz>/J) and control mice (Jackson No. C57BL/6NCrl) were intravenously injected with 200 U/kg (based on chromogenic FVIII activity) of each FVIII variant (ReFacto®, Eloctate®, AD2CD2\_SC, ADLCLD\_SC) or control solutions (Vehicle Control, 0.9% NaCl) and weight loss through bleeding, bleeding time and Hb amount by OD550 have been analyzed. Additional plasma sampling (0.5 h p.a. by retro-orbital withdraw, after experiment) have been done for analysis 10 of FVIII activity. As shown in Fig. 7, all FVIII proteins of the invention diminish total bleeding time and blood loss similar to that of control mice indicating in vivo functionality of AD2CD2\_SC and ADLCLD\_SC.

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 30 Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val  
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 Gly Arg Thr Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg  
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 His Gln Arg Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu  
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 45 Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe  
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 Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys  
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 Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr  
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	His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr			
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	Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly			
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	Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu			
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	Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala			
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15	Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg			
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	Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val			
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	Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser			
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	Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly			
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	Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg			
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	Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser			
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	Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln			
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	Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala			
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	Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala			
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	Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser			
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	Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser			
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45	Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln			
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	Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro			
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	Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro			
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	Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr			
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	Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr			
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55	Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His			
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	Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly			
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Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu  
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35     Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
35          40          45
Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
50          55          60
Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
65          70          75          80
45     Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
85          90          95
Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
100         105         110
His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
115         120         125
45     Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
130         135         140
Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
145         150         155         160
50     Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
165         170         175
Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
180         185         190
55     Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
195         200         205
Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
210         215         220
Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp

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	Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile			
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	Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser			
	290	295	300	
10	Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met			
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	Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His			
	325	330	335	
	Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro			
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15	Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp			
	355	360	365	
	Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser			
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	Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr			
	385	390	395	400
20	Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro			
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	Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn			
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	Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met			
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	Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp			
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	Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu			
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	Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp			
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	Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly			
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	Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp			
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						915			920			925				
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	Phe Ala Thr Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg		
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	Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln		
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	Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly		
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	1360		
	Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser		
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	Ser Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys		
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20	Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn		
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	Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln		
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 Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
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 245 250 255  
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 260 265 270  
 55 Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
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	Asn	Ile	Gln	Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	
							610			615			620				
	Pro	Glu	Phe	Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val	
60							625			630			635			640	
	Phe	Asp	Ser	Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	
							645			650			655				
	Tyr	Ile	Leu	Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe	
65							660			665			670				
	Ser	Gly	Tyr	Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr	
							675			680			685				
	Leu	Phe	Pro	Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	
70							690			695			700				
	Gly	Leu	Trp	Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	
							705			710			715			720	
	Met	Thr	Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	
75							725			730			735				
	Tyr	Tyr	Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	
							740			745			750				
	Asn	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Pro	Pro	Val	Leu	
80							755			760			765				
	Lys	Arg	His	Gln	Arg	Glu	Ile	Thr	Arg	Thr	Leu	Gln	Ser	Asp	Gln		
85							770			775			780				
	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile	Ser	Val	Glu	Met	Lys	Lys	Glu	

	785	790	795	800
	Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe			
	805	810	815	
5	Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp			
	820	825	830	
	Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln			
	835	840	845	
	Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr			
	850	855	860	
10	Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His			
	865	870	875	880
	Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile			
	885	890	895	
	Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser			
	900	905	910	
15	Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg			
	915	920	925	
	Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val			
	930	935	940	
	Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp			
	945	950	955	960
20	Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu			
	965	970	975	
	Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro Ala His			
	980	985	990	
	Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe			
	995	1000	1005	
25	Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys			
	1010	1015	1020	
	Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn			
	1025	1030	1035	1040
	Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly			
30	1045	1050	1055	
	Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Ile Ser Met			
	1060	1065	1070	
	Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe			
	1075	1080	1085	
35	Thr Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr			
	1090	1095	1100	
	Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile			
	1105	1110	1115	1120
	Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser			
	1125	1130	1135	
40	Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met			
	1140	1145	1150	
	Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr			
	1155	1160	1165	
	Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile			
	1170	1175	1180	
45	Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu			
	1185	1190	1195	1200
	Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln			
	1205	1210	1215	
	Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu			
	1220	1225	1230	
50	Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu			
	1235	1240	1245	
	Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile			
	1250	1255	1260	
55	Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His			
	1265	1270	1275	1280
	Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu			
	1285	1290	1295	

	Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp			
	1300	1305	1310	
	Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp			
5	1315	1320	1325	
	Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp			
	1330	1335	1340	
	Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln			
	1345	1350	1355	1360
	Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu			
10	1365	1370	1375	
	Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp			
	1380	1385	1390	
	Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe			
	1395	1400	1405	
15	Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro			
	1410	1415	1420	
	Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His			
	1425	1430	1435	1440
	Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu			
	1445	1450	1455	
20	Tyr			
	<210> 29			
	<400> 29			
	000			
25	<210> 30			
	<400> 30			
	000			
30	<210> 31			
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	<400> 35			
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	<210> 36			
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	000			
55	<210> 38			
	<400> 38			
	000			

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<210> 39
<211> 19
<212> PRT
<213> Artificial Sequence
5

<220>
<223> Thrombin-cleavable linker

<400> 39
10  Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg
      5           10           15
His Pro Ser

15 <210> 40
<211> 14
<212> PRT
<213> Artificial Sequence

20 <220>
<223> glycine-serine linker G1

<220>
<221> VARIANT
25 <222> 14
<223> G in position 14 is present or absent

<400> 40
30  Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Gly Ser Gly
      5           10
1

<210> 41
<211> 12
<212> PRT
<213> Artificial Sequence
35

<220>
<223> glycine-serine linker G2

<400> 41
40  Gly Gly Ser Gly Gly Ser Gly Gly Ser Gly
      5           10
1

<210> 42
<211> 46
45 <212> PRT
<213> Artificial Sequence

50 <220>
<223> GLG-1 linker

<400> 42
55  Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Gly Ser Lys
      5           10           15
Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro
      20           25           30
Ser Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Gly Ser
      35           40           45

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<210> 43
<211> 43
<212> PRT
<213> Artificial Sequence
5

<220>
<223> GLG-2 linker

<400> 43
10 Gly Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Lys Asn Asn
      5          10          15
Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Gly
      20          25          30
Gly Ser Gly Gly Ser Gly Gly Ser Gly
      35          40

15 <210> 44
<211> 46
<212> PRT
<213> Artificial Sequence
20

<220>
<223> ABD consensus sequence

25 <220>
<221> VARIANT
<222> 3
<223> X3 is selected from E, S, Q and C

<220>
30 <221> VARIANT
<222> 6
<223> X6 is selected from E, S, C and V

<220>
35 <221> VARIANT
<222> 7
<223> X7 is selected from A, S and L

<220>
40 <221> VARIANT
<222> 10
<223> X10 is selected from A, S and R

<220>
45 <221> VARIANT
<222> 14
<223> X14 is selected from A, S, C and K

<220>
50 <221> VARIANT
<222> 26
<223> X26 is selected from D, E and N

<220>
55 <221> VARIANT
<222> 39
<223> X39 is selected from D, E and L

<220>
<221> VARIANT

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<222> 40  
 <223> X40 is selected from A, E and H

5 <220>  
 <221> VARIANT  
 <222> 43  
 <223> X43 is selected from A and K

10 <220>  
 <221> VARIANT  
 <222> 44  
 <223> X44 is selected from A, S and E

15 <220>  
 <221> VARIANT  
 <222> 45  
 <223> L in position 45 is present or absent

20 <220>  
 <221> VARIANT  
 <222> 46  
 <223> P in position 46 is present or absent

25 <400> 44  
 Leu Ala Xaa Ala Lys Xaa Xaa Ala Asn Xaa Glu Leu Asp Xaa Tyr Gly  
 1 5 10 15  
 Val Ser Asp Phe Tyr Lys Arg Leu Ile Xaa Lys Ala Lys Thr Val Glu  
 20 25 30  
 Gly Val Glu Ala Leu Lys Xaa Xaa Ile Leu Xaa Xaa Leu Pro  
 35 40 45

30 <210> 45  
 <211> 46  
 <212> PRT  
 <213> Artificial Sequence

35 <220>  
 <223> ABD1

40 <400> 45  
 Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu Leu Asp Lys Tyr Gly  
 1 5 10 15  
 Val Ser Asp Phe Tyr Lys Arg Leu Ile Asn Lys Ala Lys Thr Val Glu  
 20 25 30  
 Gly Val Glu Ala Leu Lys Leu His Ile Leu Ala Ala Leu Pro  
 35 40 45

45 <210> 46  
 <211> 46  
 <212> PRT  
 <213> Artificial Sequence

50 <220>  
 <223> ABD2

55 <400> 46  
 Leu Ala Glu Ala Lys Glu Ala Ala Asn Ala Glu Leu Asp Ser Tyr Gly  
 1 5 10 15  
 Val Ser Asp Phe Tyr Lys Arg Leu Ile Asp Lys Ala Lys Thr Val Glu  
 20 25 30  
 Gly Val Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu Pro

35

40

45

5  
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 <211> 1575  
 <212> PRT  
 <213> Artificial Sequence

10  
 <220>  
 <223> ADLCLD\_SC aa

15  
 <400> 47  
 Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe  
 1 5 10 15  
 Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser  
 20 25 30  
 Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg  
 35 40 45  
 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
 50 55 60  
 Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
 65 70 75 80  
 Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
 85 90 95  
 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
 100 105 110  
 His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
 115 120 125  
 Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp  
 130 135 140  
 Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
 145 150 155 160  
 30 Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
 165 170 175  
 Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
 180 185 190  
 Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr  
 195 200 205  
 35 Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
 210 215 220  
 Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp  
 225 230 235 240  
 Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr  
 245 250 255  
 40 Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val  
 260 265 270  
 Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
 275 280 285  
 Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser  
 290 295 300  
 45 Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met  
 305 310 315 320  
 Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His  
 325 330 335  
 50 Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro  
 340 345 350  
 Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp  
 355 360 365  
 Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser  
 370 375 380  
 55 Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr  
 385 390 395 400  
 Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro

	405	410	415
	Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr	Leu Asn	
	420	425	430
5	Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met		
	435	440	445
	Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu		
	450	455	460
	Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu		
	465	470	475
10	Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro		
	485	490	495
	His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys		
	500	505	510
	Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe		
	515	520	525
15	Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp		
	530	535	540
	Pro Arg Cys Leu Thr Arg Tyr Ser Ser Phe Val Asn Met Glu Arg		
	545	550	555
	Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu		
	565	570	575
20	Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val		
	580	585	590
	Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu		
	595	600	605
	Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp		
	610	615	620
25	Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val		
	625	630	635
	Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp		
	645	650	655
30	Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe		
	660	665	670
	Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr		
	675	680	685
	Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro		
	690	695	700
35	Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly		
	705	710	715
	Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp		
	725	730	735
	Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys		
	740	745	750
40	Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Pro Pro Val Leu		
	755	760	765
	Ala Glu Ala Lys Glu Ala Ala Asn Ala Glu Leu Asp Ser Tyr Gly Val		
	770	775	780
	Ser Asp Phe Tyr Lys Arg Leu Ile Asp Lys Ala Lys Thr Val Glu Gly		
	785	790	795
45	Val Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu Pro Ser Lys Asn		
	805	810	815
	Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser		
	820	825	830
	Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val		
	835	840	845
50	Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln		
	850	855	860
	Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala		
	865	870	875
	Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu		
	885	890	895
55	Arg Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val		
	900	905	910

Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly  
 915 920 925  
 Glu Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu  
 930 935 940  
 5 Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro  
 945 950 955 960  
 Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln  
 965 970 975  
 Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr  
 980 985 990  
 10 Tyr Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe  
 995 1000 1005  
 Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp  
 1010 1015 1020  
 15 Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr  
 1025 1030 1035 1040  
 Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu  
 1045 1050 1055  
 Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn  
 1060 1065 1070  
 20 Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro  
 1075 1080 1085  
 Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met  
 1090 1095 1100  
 Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp  
 1105 1110 1115 1120  
 25 Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe  
 1125 1130 1135  
 Ser Gly His Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met Ala  
 1140 1145 1150  
 Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro  
 1155 1160 1165  
 30 Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu  
 1170 1175 1180  
 His Ala Gly Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln  
 1185 1190 1195 1200  
 Thr Pro Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr  
 1205 1210 1215  
 35 Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His  
 1220 1225 1230  
 Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp  
 1235 1240 1245  
 Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr  
 1250 1255 1260  
 40 Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile  
 1265 1270 1275 1280  
 Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn  
 1285 1290 1295  
 Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly  
 1300 1305 1310  
 45 Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg  
 1315 1320 1325  
 Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu  
 1330 1335 1340  
 Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser  
 50 1345 1350 1355 1360  
 Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn  
 1365 1370 1375  
 Met Phe Ala Thr Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly  
 1380 1385 1390  
 55 Arg Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu  
 1395 1400 1405  
 Gln Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln

	1410	1415	1420
	Gly Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile		
5	1425 1430 1435 1440		
	Ser Ser Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly		
	1445 1450 1455 1460		
	Lys Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val		
10	1465 1470 1475 1480		
	Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro		
	1485 1490 1495 1500		
	Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys		
15	1505 1510 1515 1520		
	Phe Ser Gln Asn Ser Arg His Pro Ser Leu Ala Glu Ala Lys Glu Ala		
	1525 1530 1535 1540		
	Ala Asn Ala Glu Leu Asp Ser Tyr Gly Val Ser Asp Phe Tyr Lys Arg		
20	1545 1550 1555 1560		
	Leu Ile Asp Lys Ala Lys Thr Val Glu Gly Val Glu Ala Leu Lys Asp		
	1565 1570 1575		
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	<211> 1778		
	<212> PRT		
	<213> Artificial Sequence		
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	<400> 48		
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	Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser		
	20 25 30		
	Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg		
35	35 40 45		
	Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val		
	50 55 60		
	Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile		
	65 70 75 80		
	Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln		
40	85 90 95		
	Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser		
	100 105 110		
	His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser		
	115 120 125		
45	Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp		
	130 135 140		
	Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu		
	145 150 155 160		
	Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser		
	165 170 175		
50	Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile		
	180 185 190		
	Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr		
	195 200 205		
	Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly		
	210 215 220		
55	Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp		
	225 230 235 240		
	Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr		

	245	250	255	
	Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val			
	260	265	270	
5	Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile			
	275	280	285	
	Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser			
	290	295	300	
	Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met			
	305	310	315	320
10	Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His			
	325	330	335	
	Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro			
	340	345	350	
	Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp			
	355	360	365	
15	Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser			
	370	375	380	
	Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr			
	385	390	395	400
	Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro			
	405	410	415	
20	Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn			
	420	425	430	
	Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met			
	435	440	445	
	Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu			
	450	455	460	
25	Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu			
	465	470	475	480
	Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro			
	485	490	495	
	His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys			
30	500	505	510	
	Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe			
	515	520	525	
	Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp			
	530	535	540	
35	Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg			
	545	550	555	560
	Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu			
	565	570	575	
	Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val			
	580	585	590	
40	Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu			
	595	600	605	
	Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp			
	610	615	620	
	Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val			
	625	630	635	640
45	Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp			
	645	650	655	
	Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe			
	660	665	670	
	Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr			
	675	680	685	
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	Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly			
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	Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp			
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55	Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys			
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	Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Pro Pro Val Leu			
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	Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg			
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	His Pro Ser Leu Ala Glu Ala Lys Glu Ala Ala Asn Ala Glu Leu Asp			
	785	790	795	800
	Ser Tyr Gly Val Ser Asp Phe Tyr Lys Arg Leu Ile Asp Lys Ala Lys			
	805	810	815	
10	Thr Val Glu Gly Val Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu			
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	Pro Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Gly Ser Gly Ser			
	835	840	845	
	Lys Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His			
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15	Pro Ser Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Gly Ser Leu			
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	Ala Glu Ala Lys Glu Ala Ala Asn Ala Glu Leu Asp Ser Tyr Gly Val			
	885	890	895	
	Ser Asp Phe Tyr Lys Arg Leu Ile Asp Lys Ala Lys Thr Val Glu Gly			
	900	905	910	
20	Val Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu Pro Ser Lys Asn			
	915	920	925	
	Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser			
	930	935	940	
	Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu			
	945	950	955	960
25	Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser			
	965	970	975	
	Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val			
	980	985	990	
	Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg			
	995	1000	1005	
30	Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe			
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	Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu			
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	Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val			
	1045	1050	1055	
35	Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr			
	1060	1065	1070	
	Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly			
	1075	1080	1085	
	Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr			
	1090	1095	1100	
40	Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp			
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	Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val			
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	His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu			
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	Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe			
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	Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met			
	1170	1175	1180	
50	Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr			
	1185	1190	1195	1200
	Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp			
	1205	1210	1215	
	Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr			
	1220	1225	1230	
55	Leu Leu Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser			
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	Gly His Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu			

	1250	1255	1260
	Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser		
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	Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His		
	1285 1290	1295	
	Ala Gly Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr		
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	Pro Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala		
	1315 1320	1325	
10	Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr		
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	Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile		
	1345 1350	1355	1360
	Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln		
	1365 1370	1375	
15	Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile		
	1380 1385	1390	
	Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser		
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	Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile		
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20	Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu		
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	His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met		
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	Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys		
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	1475 1480	1485	
	Phe Ala Thr Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg		
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	Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln		
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	Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly		
	1525 1530	1535	
	Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser		
	1540 1545	1550	
35	Ser Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys		
	1555 1560	1565	
	Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn		
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	Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln		
	1585 1590	1595	1600
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	Ala Gln Asp Leu Tyr Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser Phe		
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	Ser Gln Asn Ser Arg His Pro Ser Leu Ala Glu Ala Lys Glu Ala Ala		
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45	Asn Ala Glu Leu Asp Ser Tyr Gly Val Ser Asp Phe Tyr Lys Arg Leu		
	1650 1655	1660	
	Ile Asp Lys Ala Lys Thr Val Glu Gly Val Glu Ala Leu Lys Asp Ala		
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	Ile Leu Ala Ala Leu Pro Gly Ser Gly Gly Ser Gly Gly Ser Gly Gly		
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	Gln Asn Ser Arg His Pro Ser Gly Ser Gly Gly Ser Gly Gly Ser Gly		
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	Gly Ser Gly Ser Leu Ala Glu Ala Lys Glu Ala Ala Asn Ala Glu Leu		
	1730 1735	1740	
55	Asp Ser Tyr Gly Val Ser Asp Phe Tyr Lys Arg Leu Ile Asp Lys Ala		
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 Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg  
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 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
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 Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
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 Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
 85 90 95  
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 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
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 His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
 115 120 125  
 Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp  
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 Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
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 Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
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 Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
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 Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr  
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 Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
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 Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp  
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 Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val  
 260 265 270  
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 Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
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 Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser  
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 Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His  
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 Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro  
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## EP 3 785 726 A1

	Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr			
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5	Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn			
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10	Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu			
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	Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu			
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	His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys			
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20	Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe			
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25	Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg			
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	Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu			
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	Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu			
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	610	615	620	
	Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val			
	625	630	635	640
40	Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp			
	645	650	655	
	Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe			
	660	665	670	
	Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr			
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	Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly			
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50	Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp			
	725	730	735	
	Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys			
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	Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Pro Pro Val Leu			
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55	Gly Ser Gly Gly Ser Gly Ser Gly Ser Gly Ser Leu Ala Glu			
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	Ala Lys Glu Ala Ala Asn Ala Glu Leu Asp Ser Tyr Gly Val Ser Asp			
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	Phe Tyr Lys Arg Leu Ile Asp Lys Ala Lys Thr Val Glu Gly Val Glu			
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	Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu Pro Gly Ser Gly Ser			
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	Gly Gly Ser Gly Gly Ser Gly Ser Leu Ala Glu Ala Lys Glu Ala Ala			
	835	840	845	
	Asn Ala Glu Leu Asp Ser Tyr Gly Val Ser Asp Phe Tyr Lys Arg Leu			
	850	855	860	
	Ile Asp Lys Ala Lys Thr Val Glu Gly Val Glu Ala Leu Lys Asp Ala			
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	Ile Leu Ala Ala Leu Pro Gly Ser Gly Ser Gly Ser Gly Gly			

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	Ser Gly Ser Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile		
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	Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu		
	915 920 925		
	Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile		
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	Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His		
	945 950 955 960		
10	Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys		
	965 970 975		
	Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr		
	980 985 990		
	Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg		
	995 1000 1005		
15	Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser		
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	Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln		
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20	Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp		
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	Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu		
	1075 1080 1085		
	Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr		
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	Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe		
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	Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr		
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	Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe		
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	Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile		
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 20 His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu  
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 50 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
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 Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
 65 70 75 80  
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 85 90 95  
 55 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
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	130	135	140
5	Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu		
	145	150	155
	Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser		
	165	170	175
	Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile		
	180	185	190
10	Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr		
	195	200	205
	Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly		
	210	215	220
	Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp		
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	Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr		
	245	250	255
	Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val		
	260	265	270
20	Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile		
	275	280	285
	Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser		
	290	295	300
	Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met		
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25	Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His		
	325	330	335
	Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro		
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	Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp		
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	Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr		
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	Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro		
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	Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro		
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	Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val		
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	Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp		

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	Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp		
	645 650 655		
	Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe		
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	Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr		
	675 680 685		
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	690 695 700		
	Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly		
	705 710 715 720		
	Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp		
	725 730 735		
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	740 745 750		
	Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Pro Pro Val Leu		
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	Gly Val Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu Pro Leu Ala		
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	Glu Ala Lys Glu Ala Ala Asn Ala Glu Leu Asp Ser Tyr Gly Val Ser		
	820 825 830		
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	Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu Pro Gln Ser Asp Gln		
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	Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu		
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	Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe		
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	Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp		
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	Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His		
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	Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp		
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 Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile  
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 Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu  
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 Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro  
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 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
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 Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
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 Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
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 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
 100 105 110  
 His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
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 145 150 155 160  
 30 Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
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 Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
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 Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr  
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 Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
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 Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser  
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 50 Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro  
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 Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser  
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 Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro

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	Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu		
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	Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu		
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	His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys		
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	Pro Arg Cys Leu Thr Arg Tyr Ser Ser Phe Val Asn Met Glu Arg		
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	Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu		
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	Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp		
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	Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp		
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	Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr		
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	Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp		
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	Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys		
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	Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg		
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	Thr Val Glu Gly Val Glu Ala Leu Lys Asp Ala Ile Leu Ala Ala Leu		
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	Pro Ser Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Gly Ser Leu		
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	Gln Ser Asp Gln Glu Glu Ile Asp Phe Asp Asp Thr Ile Ser Val Glu	
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	Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg	
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	Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr	
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	Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp	
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	Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln	
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### Claims

1. A recombinant Factor VIII protein comprising a heavy chain portion and a light chain portion of Factor VIII and at least two albumin binding domains, wherein at least one albumin binding domain is C-terminal to the heavy chain portion and at least one albumin binding domain is C-terminal to the light chain portion, wherein, if the protein is a single chain protein, the albumin binding domain(s) C-terminal to the heavy chain portion is/are N-terminal to the light chain portion.
- 55 2. The recombinant Factor VIII protein of claim 1 that is a single chain protein.
3. The recombinant Factor VIII protein of any one of the preceding claims, wherein two albumin binding domains are C-terminal to the heavy chain portion and two albumin binding domains are C-terminal to the light chain portion.

4. The recombinant Factor VIII protein of any one of the preceding claims, wherein albumin-binding domains are separated from the heavy chain portion and/or the light chain portion and/or other albumin-binding domains by a linker.
5. The recombinant Factor VIII protein of claim 4, wherein the linker comprises a Thrombin-cleavable linker that optionally has the sequence of SEQ ID NO: 39.
6. The recombinant Factor VIII protein of any one claims 4 or 5, wherein the linker comprises a glycine-serine linker that optionally has the sequence of SEQ ID NO: 40 or SEQ ID NO: 41.
10. 7. The recombinant Factor VIII protein of any one claims 4 to 6, wherein said linker comprises a Thrombin-cleavable linker flanked on each side by a glycine-serine linker, wherein said linker optionally has the sequence of SEQ ID NO: 42 or SEQ ID NO: 43.
15. 8. The recombinant Factor VIII protein of any one of the preceding claims, wherein the albumin binding domain comprises a sequence according to SEQ ID NO: 44, wherein preferably the sequence is SEQ ID NO: 46.
9. The recombinant Factor VIII protein of any one of the preceding claims that optionally is a single chain protein, wherein said protein comprises a heavy chain portion having at least 90% sequence identity to aa20-aa768 of SEQ ID NO: 16 and a light chain portion having at least 90% sequence identity to aa769-aa1445 of SEQ ID NO: 16.
20. 10. The recombinant Factor VIII protein of any one of the preceding claims that is a single chain protein comprising at least two albumin binding domains between the heavy chain portion and the light chain portion and at least two albumin binding domain C-terminal to the light chain portion, wherein the protein has at least 80% sequence identity to any of SEQ ID NO: 48, 49 or 51,  
25. wherein the protein preferably has at least 80% sequence identity to SEQ ID NO: 48.
11. The recombinant Factor VIII protein of any one of the preceding claims, wherein the protein is a de-immunized protein.
30. 12. A nucleic acid encoding a recombinant Factor VIII protein of any one of the preceding claims, wherein said nucleic acid preferably is an expression vector suitable for expression of said recombinant Factor VIII protein in a mammalian cell, optionally, a human cell, such as a CAP cell.
13. A host cell comprising a nucleic acid of claim 12, wherein preferably the host cell is a mammalian cell comprising an expression vector suitable for expression of said recombinant Factor VIII protein in said cell.
35. 14. A method of preparing a recombinant Factor VIII protein, comprising culturing the host cell of claim 13 under conditions suitable for expression of the Factor VIII protein and isolating the recombinant Factor VIII protein, wherein the method optionally comprises formulating the Factor VIII protein as a pharmaceutical composition.
40. 15. A pharmaceutical composition comprising the recombinant Factor VIII protein of any of claims 1-11, the nucleic acid of claim 12 or the host cell of claim 13, optionally further comprising a biologically acceptable carrier and/or albumin, wherein the pharmaceutical composition preferably is for use in treatment of hemophilia A.

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Fig. 1

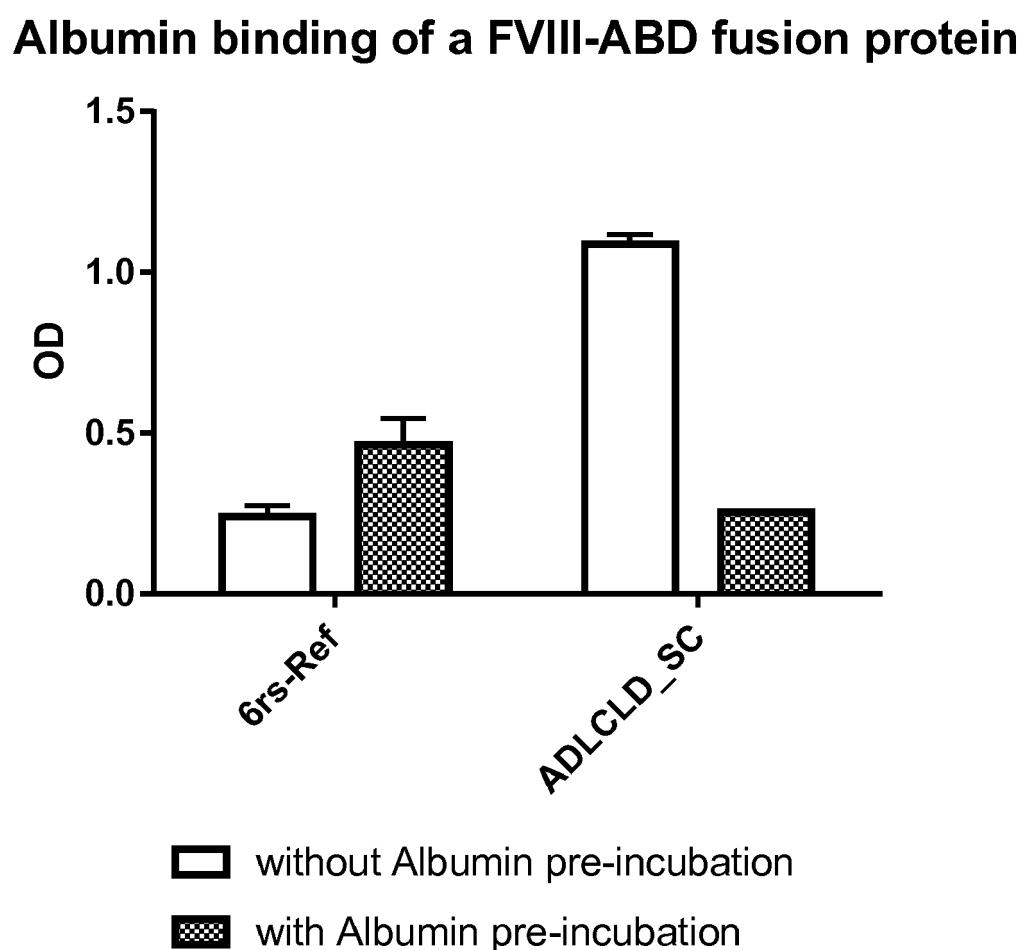


Fig. 2

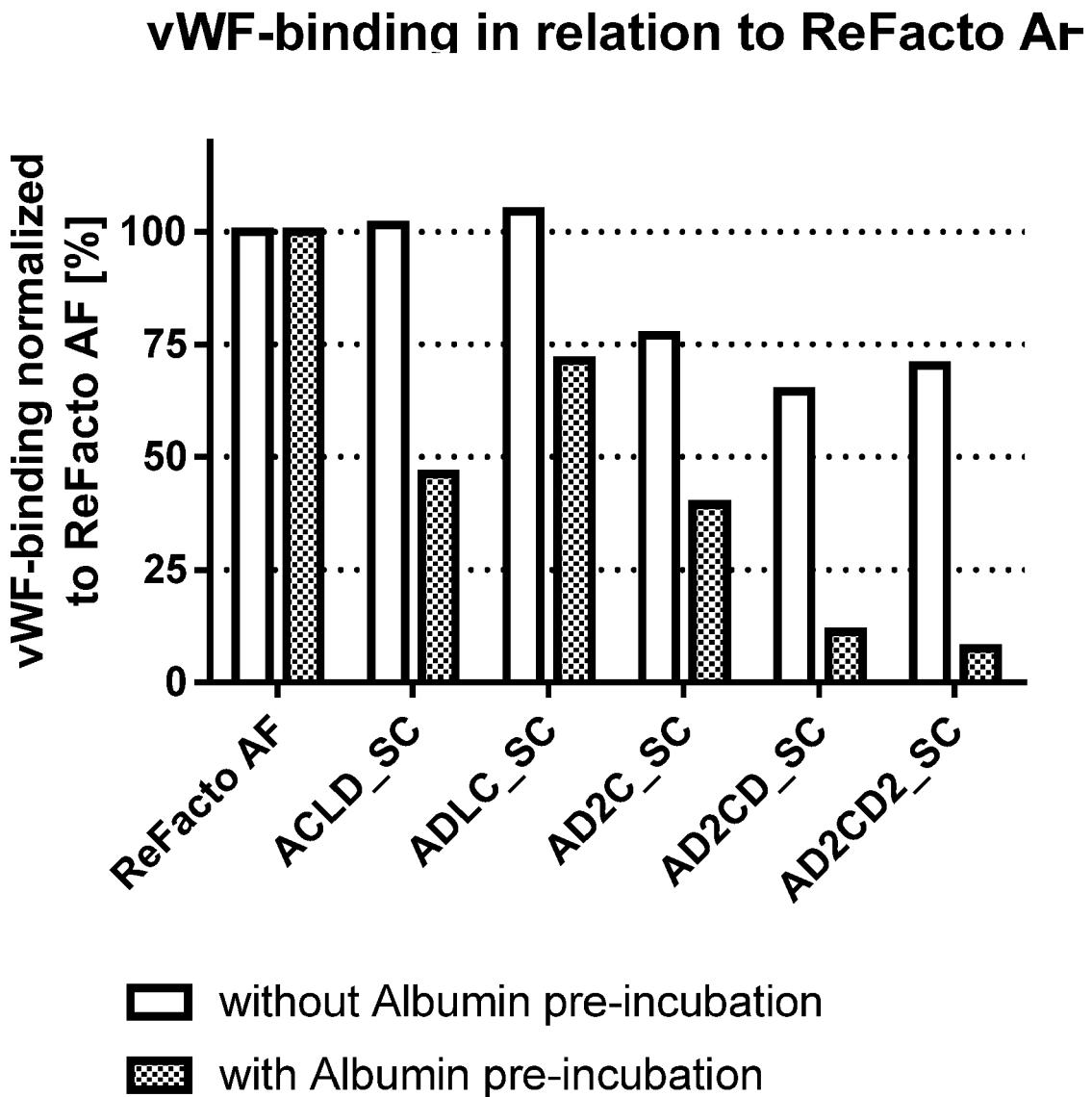


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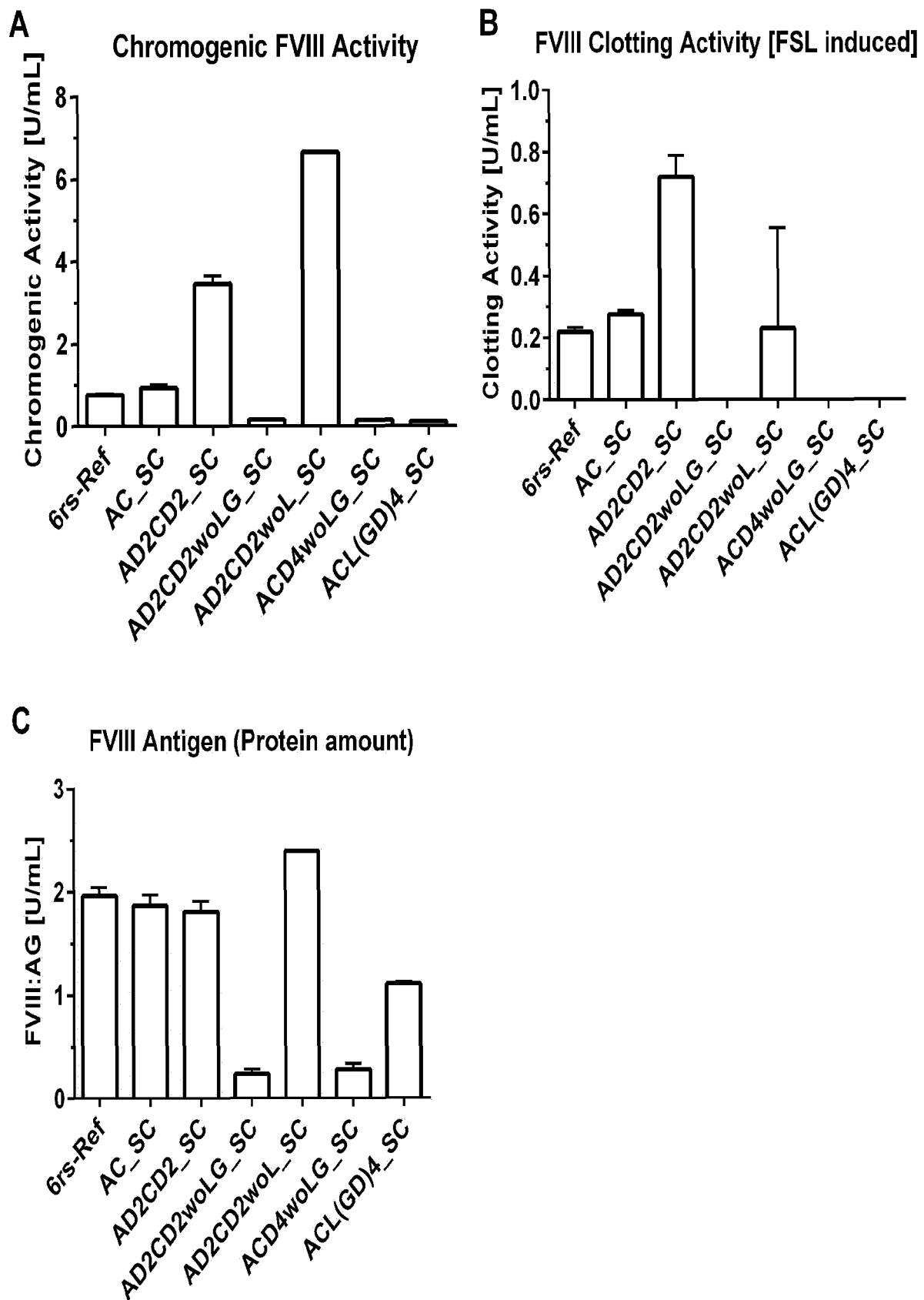


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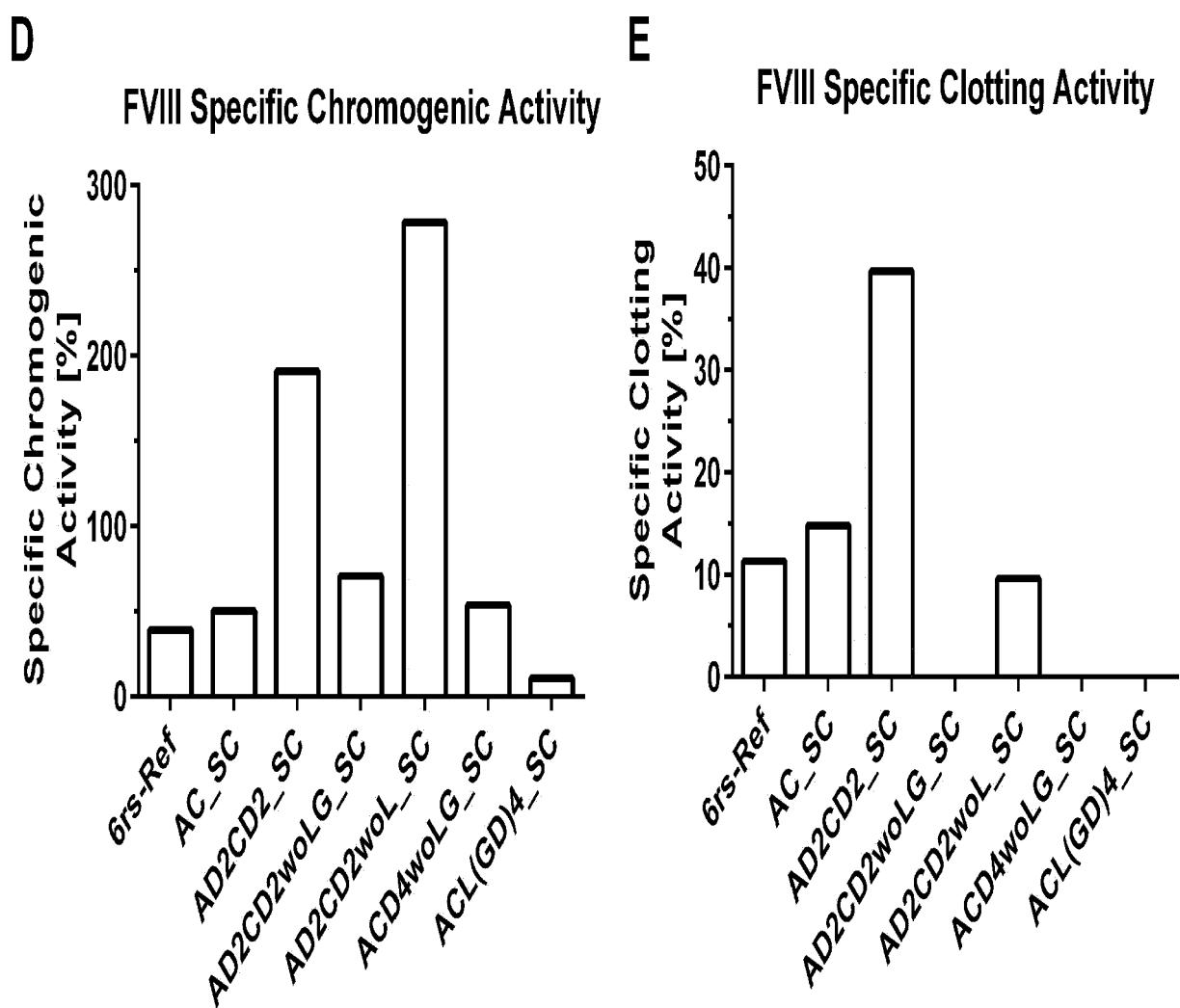


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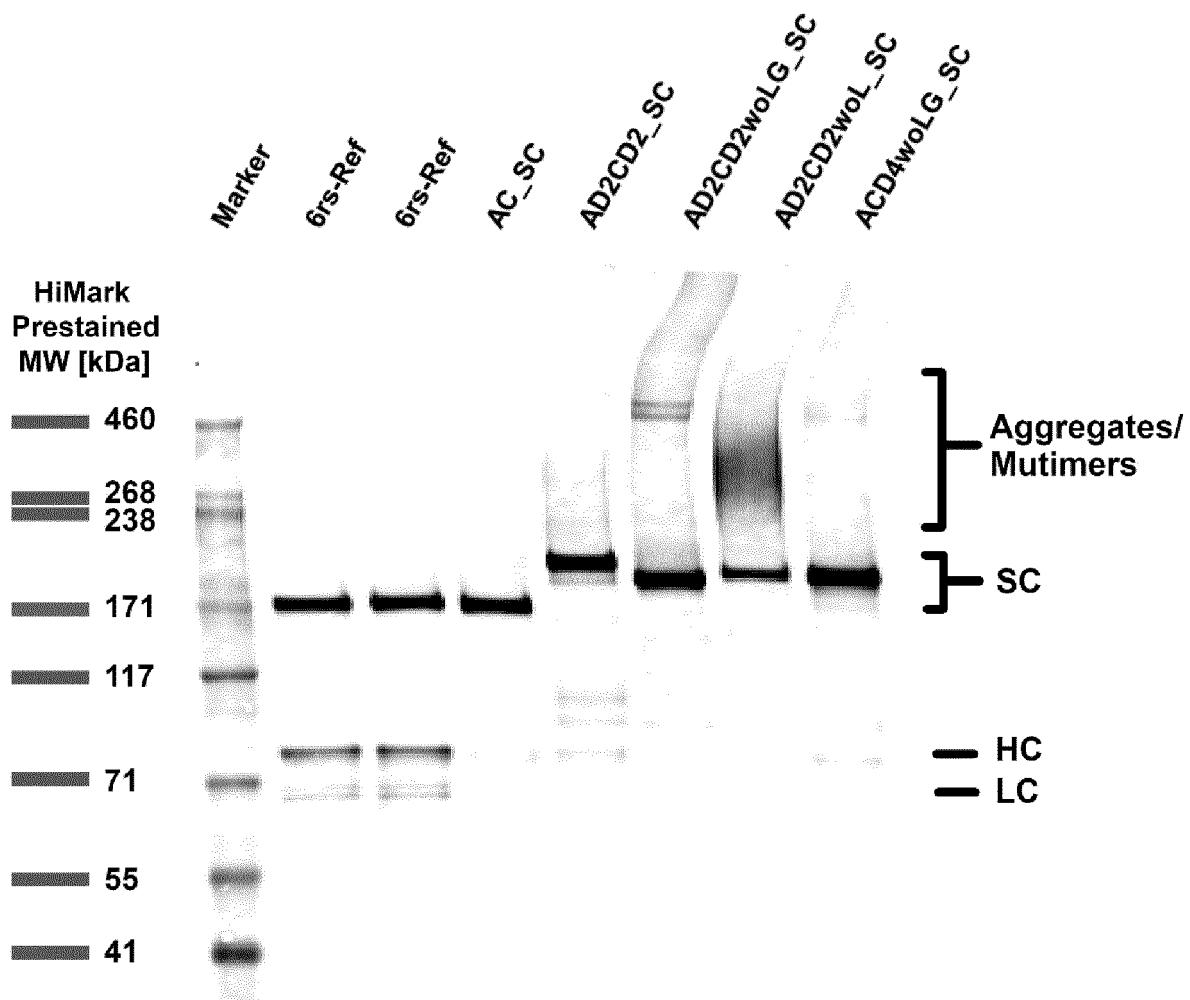


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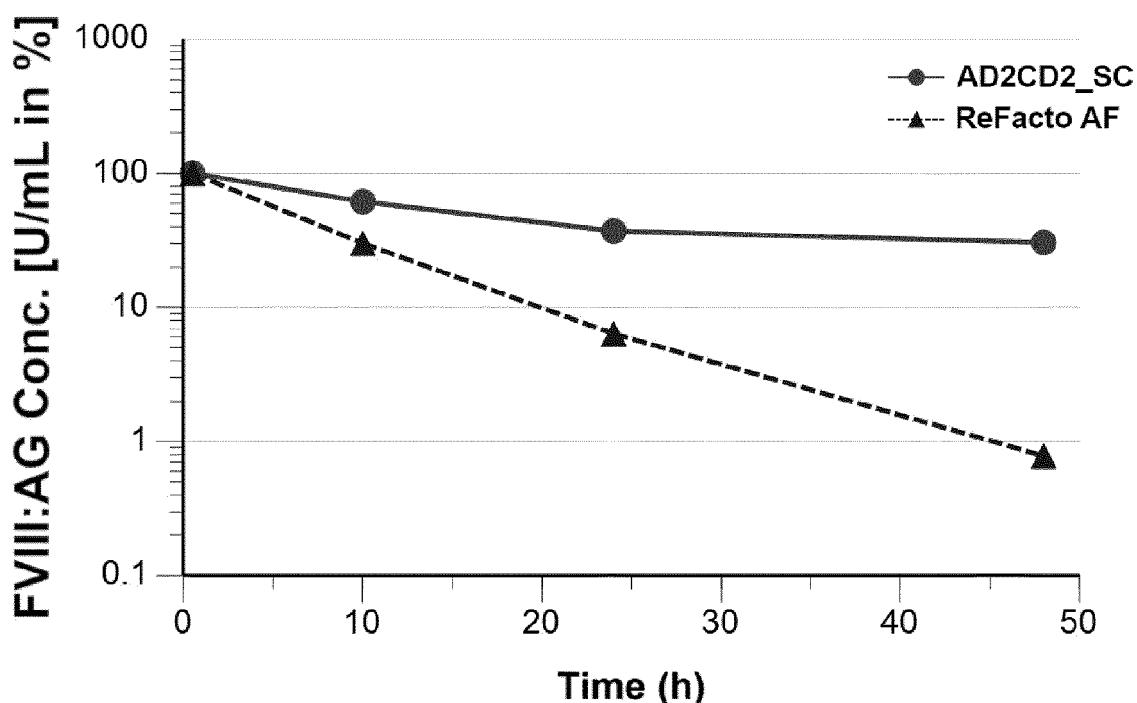


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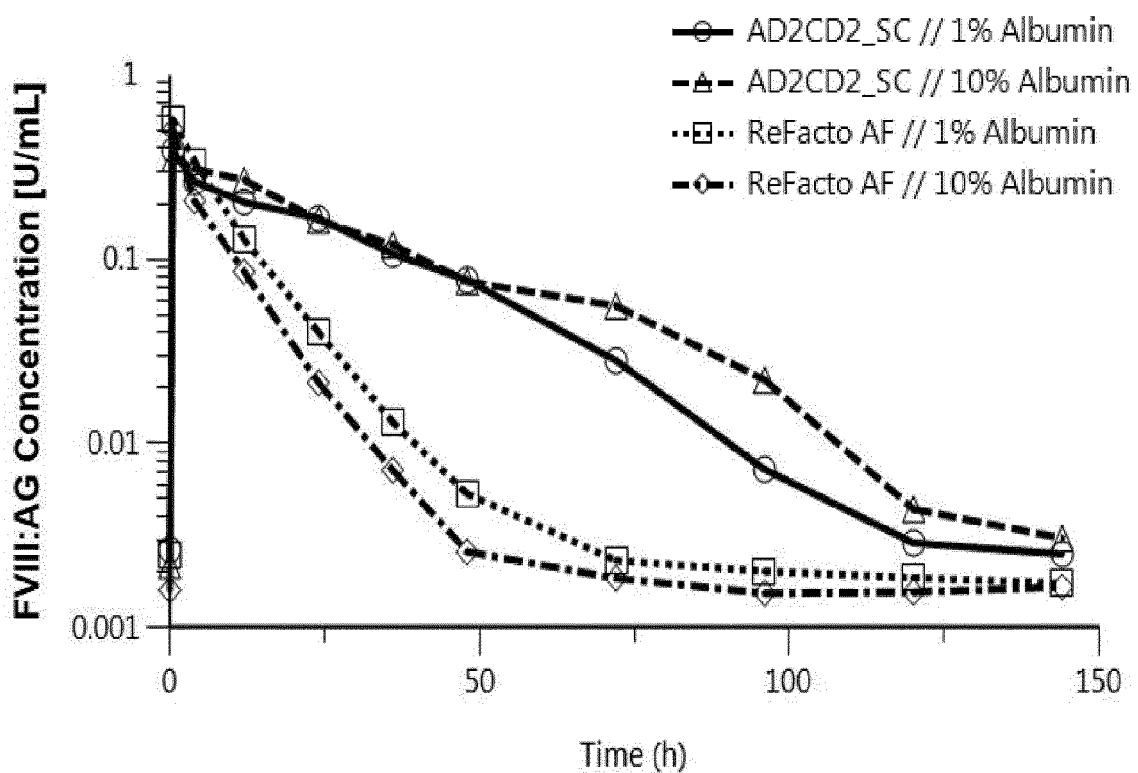
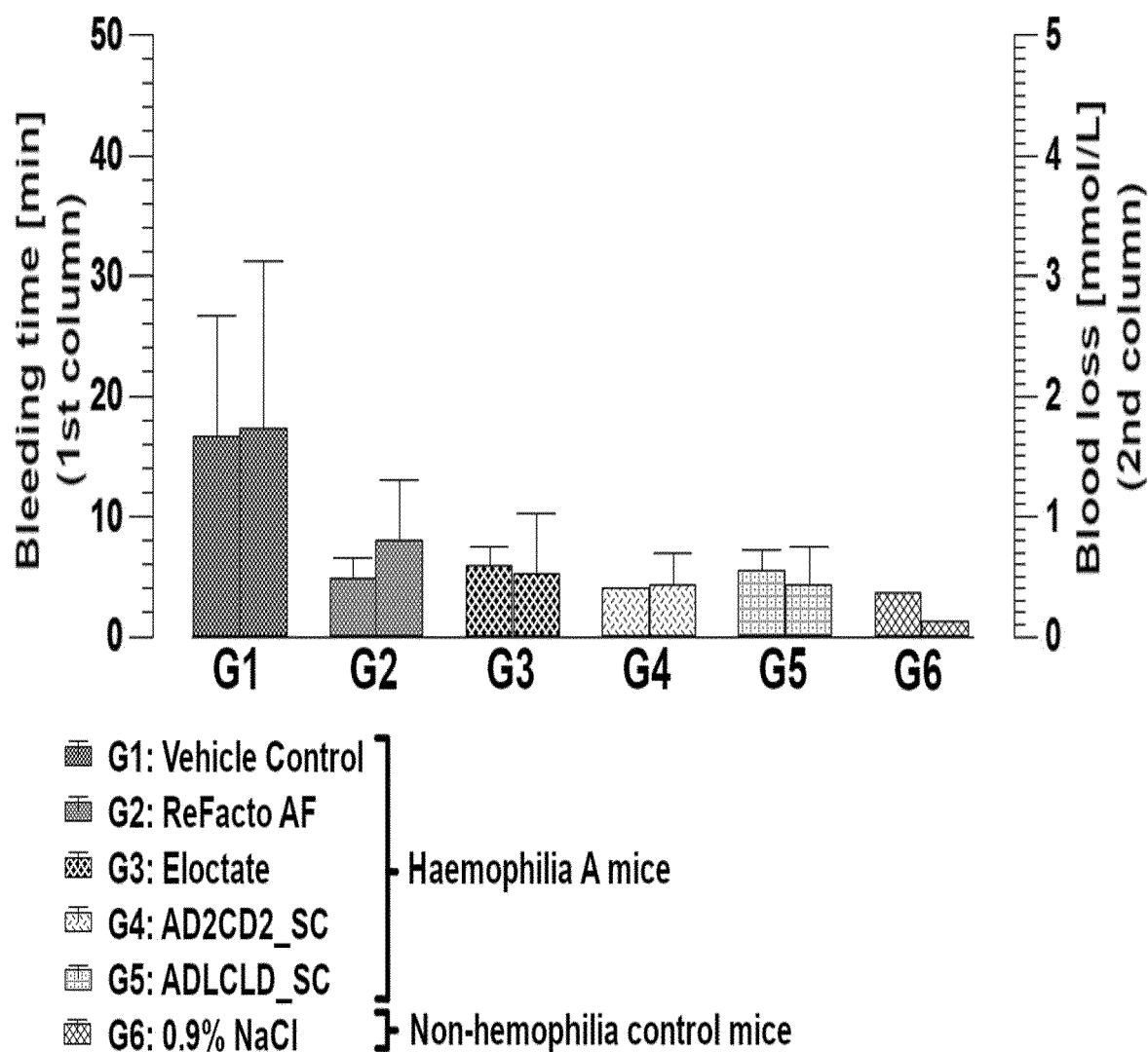


Fig. 7





## EUROPEAN SEARCH REPORT

Application Number  
EP 19 19 4964

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
10	X WO 2013/143890 A1 (AFFIBODY AB [SE]) 3 October 2013 (2013-10-03) * abstract * * page 4, line 30 - page 7, line 10 * * claims 1-17xp *	1-15	INV. A61K38/37
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